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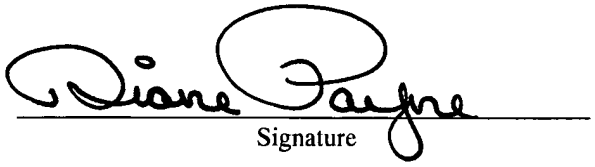

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Exhibit A – Hasan, U. *et al.*, J. Immunol. Methods 229:1-22 (1999) (23 pages)
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Exhibit C – Miyashita, M. *et al.*, Vaccine 20:2336-2342 (2000) (8 pages)
Exhibit D – Hatzifoti, C., *et al.*, Vaccine 22:2651-2659 (2004) (10 pages)

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Review

Nucleic acid immunization: concepts and techniques associated with third generation vaccines

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Abstract

A radical change in vaccine methodology arrived nine years ago with the advent of nucleic acid immunization. Aspects such as plasmid design, gene selection, the use of immunostimulatory complexes and clinical trials are discussed in this review. Furthermore, concepts and protocols involved in the construction, evaluation and immunization of a DNA vaccine have been examined as new strategies to enhance this technology continues to grow. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Nucleic acid immunization; Genetic immunization; DNA vaccines; Naked DNA; Delivery systems

1. History of DNA inoculation

The observation that DNA alone can transduce cells was first reported nearly 40 years ago, after phenol-extracted papillomavirus nucleic acid was injected into domestic rabbits and induced tumors (Ito, 1960). Subsequently Atanasiu demonstrated that the subcutaneous injection of polyomavirus DNA extracted from infected cultured cells not only induced tumors in hamsters but also generated anti-polyoma (antigen) antibodies (Atanasiu, 1962). Nearly 20 years later it was shown that polyomavirus DNA inserted into a plasmid vector, or lambda phage, was able to enter and transform murine fibroblasts (Fried et al., 1979). Although these early experiments indicated clearly the potential of DNA plasmid constructs for the delivery and expression of genes encoding a variety of proteins, there was a general belief that DNA free in the body would be rapidly degraded and be of little use for immunization.

In 1990, however, Wolff et al. (1990) reported the long-term expression of chloramphenicol acetyl transferase (CAT), luciferase, and β-galactosidase (β-*gal*) in mouse skeletal muscle after injecting purified and unadorned ("naked") plasmid DNA. Although this gene-based protein expression was not

Abbreviations: APC, antigen presenting cell; BHV, bovine hepatitis virus; CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; DC, dendritic cell; HA, haemagglutinin; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HSP, heat shock protein; HSV, herpes simplex virus; IFN, interferon; IL, interleukin; LCMV, lymphocytic choriomeningitis virus; MHC, major histocompatibility complex; NP, nucleoprotein; SV40, simian virus 40

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very efficient, it did suggest its applicability to vaccination and the concept of DNA immunization was born. Later that year the successful transfer of CAT and β-*gal* genes into rat and mouse liver, skin and muscle tissue by gold particle bombardment, with an instrument that would evolve into the "gene gun", was reported (Yang et al., 1990). Applying that technology, Tang et al. (1992) were the first to report on the development of serum antibodies against a foreign protein, after shooting gold particles, coated with plasmid DNA expressing human growth hormone, into the skin of mice. The proof of principle experiment came from Ulmer et al. (1993) who demonstrated the efficacy of a DNA vaccine in an influenza A mouse model. In this study a plasmid construct encoding the nucleoprotein (NP) of a H1N1 strain from 1934 was injected into the quadriceps muscle of mice and the animals were then challenged intranasally with a lethal dose of the H3N2 (1960) strain. 90% of the vaccinated mice survived the viral challenge and both antigen-specific cytotoxic T cells and antibodies were detected.

Nucleic acid immunization is thus the most recent approach to mobilizing the immune system against pathogenic invaders and it has rapidly evolved into the fifth route taken to produce vaccines in man and animals, following the use of live attenuated microbes, killed whole pathogens, purified whole pathogen proteins, component vaccines of immunogenic polysaccharides or sub-units, and genetically engineered live recombinant vector vaccines. The vaccine is usually a form of DNA (but sometimes RNA is used) encoding the immunogen or immunogens of interest and expressed as a protein by the

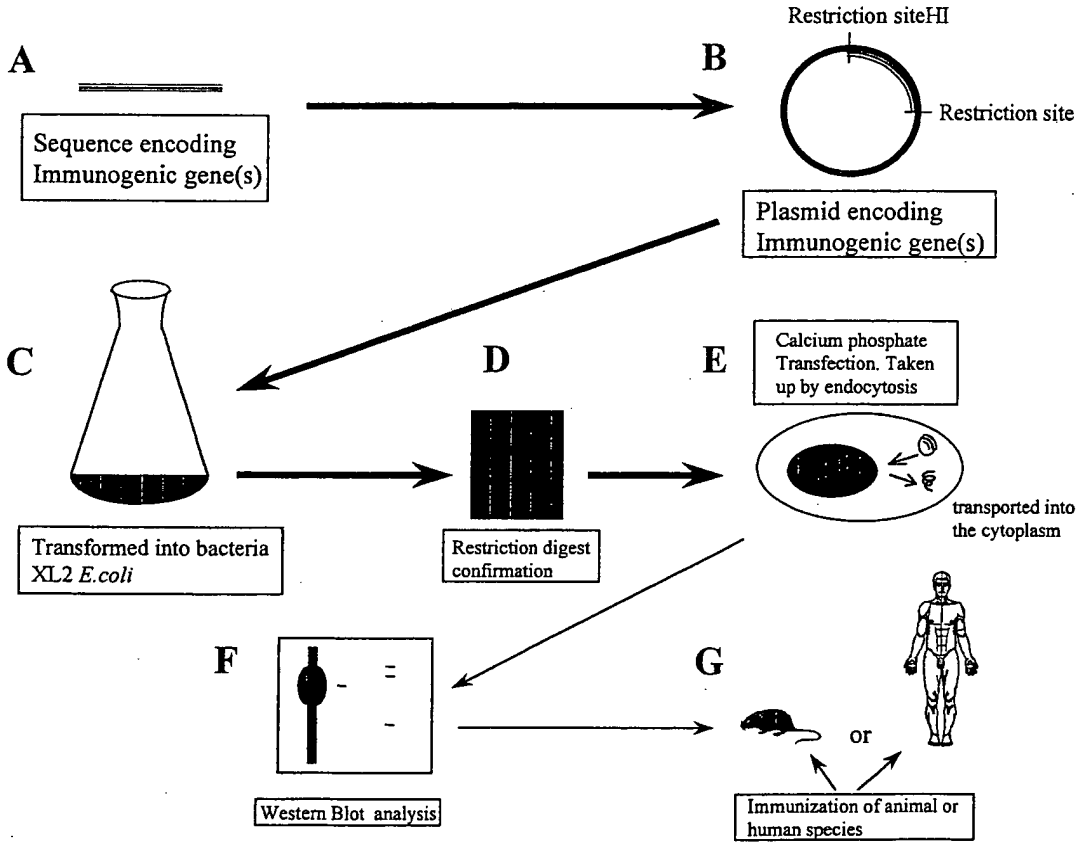

Fig. 1. Schematic diagram to illustrate the construction, evaluation and immunization of a DNA vaccine.

host cell, following inoculation of cells or tissues with its gene. Fig. 1 illustrates the production of a DNA vaccine from gene through to immunization. The term "nucleic acid immunization", with subterms DNA or RNA vaccination, is the nomenclature preferred by the World Health Organization over the description "genetic vaccination" in order to reflect the lack of intent in this technology to permanently modify the genetic make-up of the host.

Here we shall review the very rapid development of nucleic acid-based immunization that has taken place over the past few years, with emphasis on DNA vaccines against infectious pathogens, and with special reference to the practical aspects of an approach that has been heralded as the "Third Revolution" in vaccinology (Dixon, 1995).

2. Immune responses to specific pathogens

It is now well established that plasmid DNA when injected through a wide range of routes induces both humoral and cellular immune responses against the encoded immunogenic proteins in a very broad range of hosts. These responses include protective neutralizing antibodies and antigen-specific cytotoxic T lymphocytes (CTL). Responses can be boosted by repeated injections of DNA (Liu et al., 1997) and are long lasting. For example, CTL activity against influenza nuclear protein was found to persist for at least 13 months following intramuscular DNA injection in mice (Yankauckas et al., 1993).

Since the first efficacy demonstration of DNA vaccination in 1993 numerous studies of infectious diseases in animal models have been reported. Viral infections were targeted first, following the recognition that naked DNA can generate antigen-specific CTL; a response usually considered necessary for successful defence against intracellular pathogens. This particular facet of genetic immunization is one of the great advantages of the technique over conventional, protein-based vaccines. Generally, exogenous soluble proteins do not enter the MHC class I antigen processing pathway and thus cannot elicit CTL (Raychaudhuti and Morrow, 1993; Sheikh et al., 1996). This aspect is discussed in further detail in Section 10. Thus, the majority of studies to date have been carried out on viruses but parasitic diseases such as malaria, leishmaniasis, toxoplasmosis, *Taenia solium* and schistosomiasis have also been targeted, as well as diseases of bacterial origin. Tables 1A and B give an overview of which pathogen systems have been tested using DNA vaccines. It should be noted that use of the technique is not necessarily confined to vaccine development: DNA immunization of mice has been used for the successful production of monoclonal antibodies (Barry et al., 1994).

3. Vectors

The first successful demonstration of nucleic acid vaccination was carried out with plasmid DNA and apart from sporadic efforts with RNA, plasmid DNA has remained the vector of choice for this technology. However, several recent publications have described a novel way of enhancing the efficacy of DNA vaccines. Instead of using plasmid DNA grown in and purified from *Escherichia coli*, bacteria themselves carrying DNA expression plasmids have been used directly as vaccination vectors. Aspects of these techniques are examined in detail in this Section.

3.1. Plasmid DNA

Bacterial plasmids are autonomous units that exist in the cell as extrachromosomal genomes. They are self-replicating circular molecules of DNA that are maintained in the cell in a stable and characteristic number of copies. Bacterial plasmids can reproduce even after additional sequences of DNA have been incorporated into their genomes and therefore chimeric elements can be obtained in large amounts from the progeny. Plasmid DNA for vaccination purposes is engineered to carry an insert comprising sequences for the protein to be used for immunization as well as control elements, such as a eukaryotic promoter and a poly (A) site, that allow expression of the protein in eukaryotic cells. Plasmid DNA is mostly grown in and purified from *E. coli* (Horn et al., 1995) and generally made to the desired concentration using PBS or saline as the carrier vehicle.

A limitation to be addressed in the preliminary design of a DNA vaccine is the size of the construct. With the development of plasmid cloning vehicles over the last 25 years, current vectors tend to be smaller in size in order to accommodate foreign DNA with a wide range of restriction sites. Smaller

Table 1
(A) DNA vaccines for infectious diseases
I.M = intramuscular, I.D = Intradermal, I.P = Intraperitoneal, I.V = Intravenous, I.N = Intranasal, S.C = Subcutaneous.

	Host	Route	Challenged	Immune response	Protection	Refs.
Viral antigen						
Nucleoprotein of LCMV	Mice	Abdominal	Yes	Antibodies	Partial	Kriesel et al. (1996)
Nucleoprotein of LCMV	Mice	Gene gun	Yes	CTL	Partial	Zarozinski et al. (1995)
Nucleoprotein of influenza	Mice	I.M, I.V, I.N, S.C, I.P, gene gun	Yes	Antibodies and CMI	Yes, from most routes	Fynan et al. (1993)
Nucleoprotein of influenza	Mice	I.M	Yes	Antibodies and CTLs	Yes	Ulmer et al. (1993)
HBV-hepatitis B	Mice	I.M	No	Antibodies and CTLs	N/A	Davis et al. (1995)
Rabies G protein	Mice	I.M	Yes	Antibodies and CTL	Yes	Ertl and Xiang (1996)
Nucleoprotein of influenza	Mice	I.D	Yes	Antibodies and CTL	Yes	Rhodes et al. (1994)
HIV-1gp160/rev, gp120 and rev	Mice and monkeys	I.M and I.D	No	Antibody and CMI	N/A	Shiver et al. (1995)
HIV-1 Env, gag/pol genes	Chimpanzees	I.M	Yes	Antibodies and CTL	Yes	Boyer et al. (1997)
HIV-1 Env DNA and protein	Rhesus monkeys	I.M	Yes	Antibodies, Th and CTL	Yes	Letvin et al. (1997)
FIV env	Cats	I.M	Yes	Antibodies increased infection	No	Richardson et al. (1997)
HIV-1 Env, and rev	Mice, rabbits and monkeys	I.M	No	Antibodies and CTL	N/A	Okuda et al. (1995)
EHV gp 14	Mice	I.M and I.N	Yes	Humoral and DTH	Partial	Osterrieder et al. (1995)
Hep C genes + IL-12 and IL-4	Mice	I.M	No	CD4 and CD8	N/A	Manickan et al. (1997)
Influenza NP mutant	Mice	I.M	Yes	CTL	Yes	Fu et al. (1997)
Encephalitis viral gene	Mice	Gene gun	Yes	Antibodies	Partial	Schmaljohn et al. (1997)
BHV-1 gp-Bovine Herpes Virus	Cattle	I.M	No	Antibodies	N/A	Cox et al. (1993)
SIV Env and Gag	Monkeys (Macaques)	I.V, I.M, gene gun	Yes	Antibodies and CTL	Yes	Lu et al. (1996)
Major gene from Papilloma virus	Rabbits	I.M	Yes	Antibodies	Yes	Donnelly et al. (1995, 1996)
F-protein Newcastle disease virus	Chicken	I.M	Yes	Antibodies	Yes	Sakaguchi et al. (1996)
ppu188-CMV (human)	Mice	I.M	No	Antibodies	N/A	Pande et al. (1995)
gp53-Bovine virus	Mice	I.M, I.D	No	Antibodies	N/A	Harpin et al. (1997)
Foot and mouth disease gene	Mice (swine)	I.D and I.M	Yes	Antibodies	Partial	Ward et al. (1997)
Ebola nucleoprotein and gp	Mice and guinea pigs	I.M	Yes	Antibodies and CTL	Partial	Xu et al. (1998)
Hep B pre and surface ags	Peking ducks	I.M	Yes	Antibodies	Limited	Triyatni et al. (1998)
Measles HA and NP	Mice	Gene gun	No	Antibodies	N/A	Cardoso et al. (1998)
Pseudorabies glycoprotein	Mice	I.M	Yes	Antibodies	Yes	Ho et al. (1998)
Sendai virus epitopes	Mice	Gene gun	Yes	CTL	Partial	Chen et al. (1998a,b)
Foot and mouth disease capsid	Mice	Gene gun	No	Antibodies	N/A	Chinsangaram et al. (1998)

Rabies glycoprotein	Neo and preg mice	I.M	Yes	Maternal antibodies	No	Wang et al. (1998)
Influenza A HA	Pigs	Gene gun and mucosal	Yes	Antibodies	No	Macklin et al. (1998)
Influenza A HA	Mice	I.M	Yes	Antibodies	Partial	Antohi et al. (1998)
HIV-1 *nef*, *rev* and *tat* genes	Humans	I.M	No	CTL	N/A	Calarota et al. (1998)
HIV-1 *env* and *rev* genes	Humans	I.M	No	Antibodies and CTL	N/A	Magregor et al. (1998)
HIV-1 synthetic gp 120 seq	Mice	I.M	No	Antibodies and CTL	N/A	Andre et al. (1998)
HIV-1 *vif*, *vpr*, *vpu* and *nef* genes	Mice	I.M	No	Antibodies and CMI	N/A	Ayyavoo et al. (1998)
HCV, NS3, NS4 and NS5 genes	Mice	I.M	No	Antibodies and CMI	N/A	Encke (1998)
HSV-1, gC, gD, gE	Mice	I.M	Yes	Antibodies	Partial	Nass et al. (1998)
HCV env and GMCSF	Rats	I.M	No	Antibodies	N/A	Lee et al. (1998)
HIV-1 *env* and *rev*	Mice	I.M and I.N	No	Antibodies and CTL	N/A	Hamajima et al. (1998)
gP E Varicella-Zoster Virus	Mice	I.M	No	Antibodies	N/A	Hasan et al. (1997)
Bacterial Antigen						
Mycobacterium TB ELI	Mice	I.M	Yes	Antibodies and T Helper	Yes	Barry et al. (1995)
Mycobacterium TB-hsp65 gene	Mice	I.M	Yes	T helper and antibodies	Same as BCG immune response	Tascon et al. (1996)
Mycobacterium TB 38-kDa gene	Mice	I.M	Yes	CMI	Yes	Zhu et al. (1997)
Tetanus toxin - frag C	Mice	I.M	No	Antibodies	N/A	Anderson et al. (1997)
OspA - *Borrelia burgdorferi*	Mice	I.M	Yes	Antibodies	Yes	Luke et al. (1997)
E. coli CFA gene	Mice	I.M	No	Antibodies	N/A	Alves et al. (1998)
Mycobacterium TB Hsp65	Mice	I.M	Yes	CMI	Yes	Bonato et al. (1998)
Parasitic Antigen						
Malaria circumsporozoite protein	Mice	I.M	Yes	CTL, cytokines and nitric oxide	Partial	Doolan et al. (1996)
Schistosoma japonium	Mice	I.M	No	Antibodies	N/A	Yang et al. (1995)
Leishmania major gp63	Mice	I.M	Yes	IL-2 and IFN gamma	Yes	Xu and Liew (1995)
Malaria circumsporozoite protein	Mice	I.M and gene gun	Yes	Antibodies	Decreased infection	Leitner et al. (1997)
Taenia solium (KETc7)	Mice	I.M	Yes	Antibodies	Yes	Rosas et al. (1998)
Leishamania major LACK ag	Mice	I.D	Yes	Antibodies and CMI	Yes	Gurunathan et al. (1997)
Malaria, 10 peptide sequences	Human	I.M	No	CTL	N/A	Wang et al. (1998)
T. cruzi, TSA-1	Mice	I.M	Yes	Antibodies and CTL	Partial	Wizel et al. (1998)
Leishmania gp 63	Mice	I.D	Yes	Antibodies and CMI	Partial	Walker et al. (1998)
Taenia crassiceps, KETc-T	Mice	I.M	Yes	Antibodies	Partial	Rosas et al. (1998)
Taenia crassiceps, ELI	Mice	I.M and S.C	Yes	CMI	Partial	Manoutcharian et al. (1998)
Malaria gene with subunit and recombinant proteins	Mice	I.M	Yes	CMI	Yes	Schneider et al. (1998)

Table 1 (continued)
(B) DNA vaccines for other diseases

Allergy	Host	Refs.
Autoimmune disease, plasmid DNA	Mice	Mor et al. (1997)
Adjuvant arthritis, mycobacterium hsp 65	Rat	Ragno et al. (1997)
Hose Dust Mites, allergen (Der p5)	Rat	Hsu et al. (1996)
Systemic Lupus Erythematosus, TGFβ delivery and IL-2	Mice	Raz et al. (1995)
Cancer	**Host**	**Refs.**
SV40 virus, large T-antigen	Mice	Schirmbeck et al. (1996)
Renca tumor cells IL-12	Mice	Tan et al. (1996)
Proto-oncogene, p185erbb2, neu	Mice	Concetti et al. (1996)
Melanoma cells MAGE-1, MAGE-3 and GM-CSF and B7-1	Mice	Bueler and Mulligan (1996)
B cell lymphoma, idiotype	Mice	Hakim et al. (1996)
Renca tumor cells, hIL-6, TNF, mIFN gamma and mIL-12	Mice	Sun et al. (1998)
Metastic murine tumours, IL-12	Mice	Rakhmilevich et al. (1996)
CNS tumours, dendritic cells pulsed with tumour extracts	Mice	Ashley et al. (1997)
CNS, AVP gene	Rat	Geddes et al. (1997)
Melanoma, fibroblasts transfected with DNA from melanoma	Mice	de Zoten et al. (1998)
B cell lymphoma, Fv fragment idiotyes	Humans	Hawkins et al. (1997)
Experimental autoimmune encephalomyelitis, mylein basic protein DNA targeted to Fc region	Rats	Lobell et al. (1998)
Oncogenic HTLV-1	Mice	Agadjanyan et al. (1998)
Anti tumor CTL, epitopes; mutant p53, HIV gp120	Mice	Ciernik et al. (1996)
Mammory tumors, extracellular domain, extracelluar, transmembrane domain and full length neu DNA	Mice	Chen et al. (1998a,b)

plasmids are preferred for many reasons. A rule of thumb is that the efficiency of transformation is inversely related to the size of the plasmid, which becomes a limiting factor when the construct exceeds approximately 15 Kb. Therefore smaller plasmids are desirable as they can accommodate larger segments of DNA before the efficiency of transformation begins to deteriorate. Larger plasmids are also more difficult to characterize by restriction mapping and replicate too low copy numbers thereby reducing the yield of DNA.

3.1.1. Promoter selection and vector optimization

An important criterion for the success of DNA vaccination is achieving adequate levels of expression of the vector-encoded protein. Plasmid DNAs driven by viral promoters with broad cell type specificity have remained the most frequently used vehicle of DNA vaccination. In particular the cytomegalovirus immediate early (CMV-IE) and the RSV promoters, due to the fact that they have been shown to generate consistently high levels of protein expression.

A CMV promoter-driven expression vector for an influenza virus nuclear protein (NP) stimulated higher specific antibody responses than did a vector with a Rous sarcoma virus (RSV) promoter in mice vaccinated by intradermal gene immunization, contrary to the finding that the vectors induced an equivalent immune response after i.m. injection (Raz et al., 1994). In another direct comparison, a CMV-IE promoter driven expression vector for luciferase yielded about three times higher expression of this protein than the same vector driven by the RSV promoter.

An improved version of the CMV-IE promoter-driven-plasmid DNA expression vector has been developed by a series of modifications to the existing polyadenylation and transcriptional termination sequences, plasmid backbone elements and the luciferase reporter gene sequence itself. These modifications consisted of: (i) removal of all SV-40 derived sequences, including the terminator and the T-antigen dependent viral origin of replication, (ii) inclusion of a minimal transcriptional terminator from the rabbit β-globin gene (containing the essential poly-A signal and downstream termination elements but lacking any upstream 3′ UTR sequence) and (iii) expression of a modified luciferase gene (Luc +) targeted to the cytoplasm rather than to the peroxisomes. The resulting final vector produced 300 ng of luciferase at 7 days post intramuscular injection of 50 μg of DNA: an expression level 46-fold higher than achieved with the parent vector. Expression levels were 22-fold higher without the modifications to the luciferase gene. (Hartikka et al., 1996; Liang et al., 1996; Norman et al., 1997).

Selection of the most suitable promoter may depend not only on the type of cell and tissue used for expression but also on the nature of the encoded protein. Xiang et al. (1995) demonstrated protective immunity against rabies virus in mice immunized intramuscularly with a plasmid vector expressing the full-length rabies virus glycoprotein (G protein) under the control of the simian virus 40 (SV40) promoter. In vitro expression of the glycoprotein in cultured cells could also be demonstrated upon transfection with the vector driven by the SV40 promoter. Replacing the SV40 promoter of the original vector with the CMV-IE but leaving other parameters of the plasmid intact resulted in a vector that gave only a very transient in vitro expression of the rabies A protein. Thus, the two vectors showed a striking difference in their ability to cause stable expression of the rabies virus G protein in vitro, yet induced comparable immune responses following inoculation into mouse muscle in vivo. This finding most probably reflects the toxicity of this protein upon over-expression achieved by the very potent CMV promoter in cultured cells in vitro.

In addition to viral promoters, the use of mammalian house-keeping or tissue-specific promoters has also been studied. The β-actin, α-globin, MCK or MHC promoters may be less efficient in driving protein expression, but at the same time they have been shown to be less prone to "promoter-shut-off" than viral promoters (Harms and Splitter, 1995; Qin et al., 1996).

3.2. Attenuated bacterial vectors

A handful of recent publications have described a novel way of enhancing the efficacy of DNA vaccines. Instead of using plasmid DNA grown in and purified from *E. coli*, bacteria themselves carrying DNA expression plasmids have been used directly as vectors for vaccination. With appropriately chosen

bacteria, functional DNA is delivered inside infected cells. Sizemore et al. (1995, 1997) used an auxotrophic Shigella strain carrying the *E. coli* β-galactosidase gene to vaccinate mice intranasally and subsequently showed serum antibodies and a lymphoproliferative response to β-galactosidase in these animals. Darji et al. (1997) grew a plasmid carrying *Listeria monocytogenes* antigens in a mutant strain of *Salmonella typhimurium* and gave the transfected salmonella as an oral vaccine to mice. The salmonella crossed the gut wall and infected phagocytes that then expressed the DNA-encoded antigens and elicited a protective immune response against *L. monocytogenes*. Dietrich et al. (1998) have reinforced the validity of this technique by demonstrating that an attenuated strain of *L. monocytogenes* can be used to deliver defined reporter genes.

4. Antigen

Nucleic acid immunization offers enormous opportunities for genetic tailoring of antigens and rational vaccine design. In theory, selected antigens such as B and T cell epitopes and neutralization sites can be selected and inserted into the plasmid as required to give over-expression in comparison with the native antigen. While some investigators have made use of bicistronic constructs most investigations conducted to date have focused on the use of a gene encoding a major defined antigenic protein of the micro-organism in question. While this approach may be considered simplistic it does, nevertheless, have the advantage of presenting large numbers of B and T cell epitopes (both known and unknown) to the immune system in an empirical fashion.

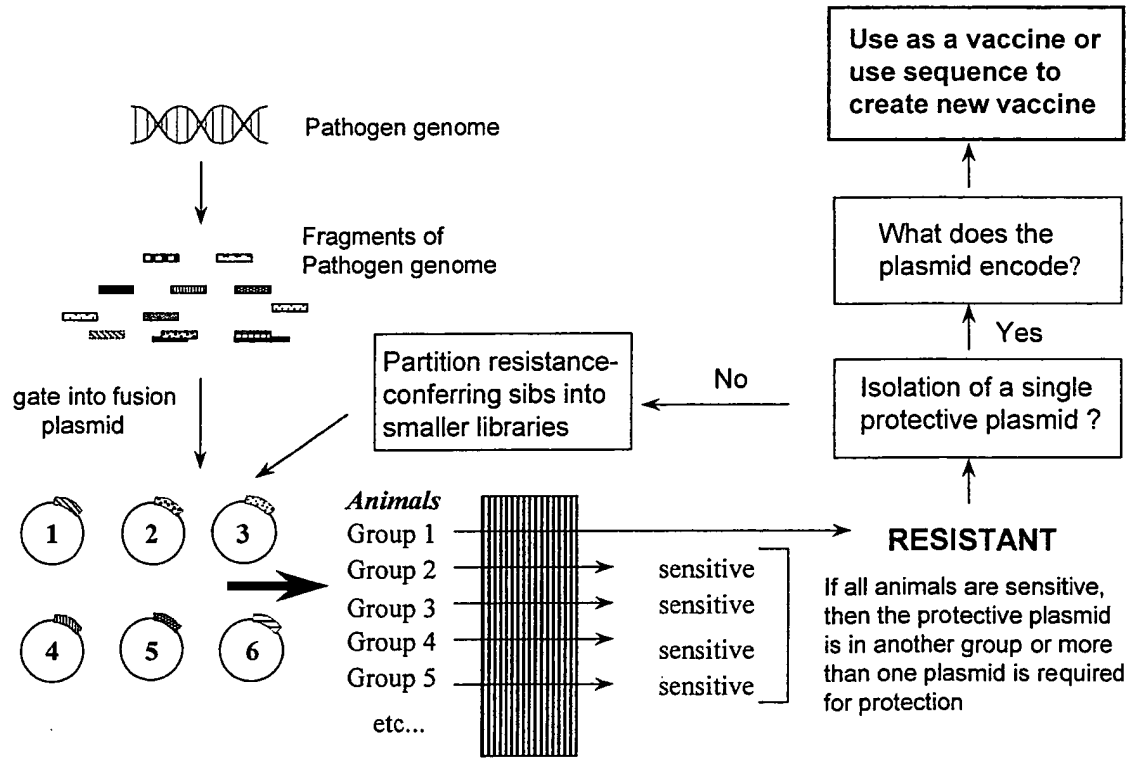

Fig. 2. The basis of Expression Library Immunization (ELI). This approach makes use of DNA vaccination by immunizing an expression library containing the entire genome of a selected pathogen. The genes conferring protection can be isolated, identified and investigated. This technique is useful when the antigens conferring protection are unknown. The technique is limited if the pathogen has a large genome. Figure adapted from Johnston and Barry (1997).

A fascinating approach described by Johnston and Barry (1997) illustrates a very powerful approach to antigen selection. The strategy known as expression library immunization (ELI) is based on the concept that the DNA encoding the entire genome of a known pathogen can be cut up and incorporated into a relatively small number of plasmids. The plasmids are tested for their ability to protect mice against the pathogen in question. If a plasmid is found to induce protective immunity, the genes it encodes are sub cloned into a further series of plasmids which in turn are tested for their immunization potential. The process is repeated until the genes encoding the protective epitopes are identified (see Fig. 2). This technique has been used to identify defined nucleic acid vaccines for *Mycoplasma pulmonsis* (Barry et al., 1995) and *Taenia crassiceps* (Manoutcharian et al., 1998). The limitations of ELI are that it requires a murine model of the disease and that it is restricted by the genomic size of the pathogen. While bacteria and viruses are of manageable size, organisms such as protozoan parasites have genomes which are too large to make the evaluation of the multiple plasmids a realistic proposition.

5. Means of delivery

The main method of delivery used for the majority of studies in Tables 1A and B is the use of needle injection to deliver DNA to the muscle and different layers of the skin. This technique is inexpensive and, most importantly, a historical method of delivering vaccines. Particle bombardment by means of a "gene gun" is another method of inoculating DNA primarily into the skin. This device utilizes a shock wave generated by compressed helium to accelerate DNA-coated gold particles directly into the cells of target tissues. Even at submicrogram quantities of DNA per dose, thousands of DNA copies can be introduced into the cells of vaccinated tissues, resulting in high level transgene expression (Rakhmilevich et al., 1996). DNA delivered cutaneously via a gene gun is targeted to cells in the epidermis. Epidermal Langerhans cells may play an important role in antigen presentation and the immunocompetence of the skin (Pertmer et al., 1996). Fynan et al. (1993) found that while at least 100 μg of purified DNA was required to protect animals from influenza virus when DNA was inoculated with a syringe, only as little as 0.4 μg of the same DNA was needed to achieve the same effect when it was coated onto gold beads and propelled with a gene gun. Furthermore Livingston et al. (1998) used a gene gun to deliver DNA encoding human growth hormone to the vaginal mucosa. This route yielded higher levels of cervicovaginal antibodies than did DNA delivery to the skin or Peyer's patches. Thus, the gene gun system makes it possible to use very small quantities of DNA to induce an immune response without the requirement for an infectious agent vector or adjuvant.

6. Sites of inoculation

6.1. Skeletal muscle

The first successful and the most frequently targeted DNA delivery site is skeletal muscle, using a syringe and needle injection approach and saline as the vehicle. Successful vaccination of mice by intramuscular needle injection usually requires DNA doses of microgram quantities. However, several groups have reported that the efficiency of gene transfer is at least ten times higher in regenerating muscle than in normal mature muscle. Controlled damage and regeneration of muscle tissue can be induced by injection of certain snake toxins such as cardiotoxin and notoxin. Such agents have been used to facilitate uptake of DNA in mice and rats, thereby increasing the numbers of inflammatory cells, which results in better presentation of the DNA (Davis et al., 1993). However, the lack of APCs in muscle tissue has caused many groups to speculate on the uptake mechanism of DNA (see below).

6.2. Skin

When considering routes of immunization, the routes of infection of the target pathogen, most notably the anatomical sites where the agent is first encountered by the immune system, must be considered. It is thus informative to observe the type of immune responses obtained from delivery of DNA via different routes of inoculation. Pertmer et al.

(1996) showed that endpoint immunoglobulin G (IgG) CTL activities were identical between mice immunized via the intramuscular and epidermal (gene gun) routes with 100 μg and 1 μg, respectively, of an influenza virus nucleoprotein (NP) expression vector. Muscle inoculation resulted in predominantly IgG2a responses, whereas gene gun inoculation yielded a preponderance of IgG1 antibodies. Although these data suggest that muscle and gene gun delivery elicite Th1- and Th2-like responses respectively, interferon-γ (IFN-γ) profiles from antigen stimulated splenocytes were remarkably similar between these groups. IL-4 production assays revealed qualitative differences that could be correlated with the divergent IgG subclass, as there were high levels of IL-4 in the gene gun inoculations whereas levels of IL-4 were not observed in the intramuscular DNA inoculation group. Feltquate et al. (1997) showed the opposite by utilizing an influenza haemagglutinin (H1)-expressing plasmid. They compared the immune response produced by saline injection of DNA into skin or muscle, and gene gun immunization of skin or muscle. They found that saline-DNA immunization raised a predominantly Th1 response with mostly IgG2a anti-H1 antibodies, while gene gun DNA immunization produced a predominantly Th2 response with mostly IgG1 anti-H1 antibodies. These distinct types of immune responses were generated by the method, not the route, of DNA immunization. The initial immunization established the Th cell-type of the immune response. The Th cell-type did not change with further DNA immunizations of the same type or after a viral challenge. Furthermore, the ability to generate different Th types was not due to differences in the doses of DNA used in the saline and gene gun DNA immunizations.

Although striated muscle has been suggested to be unique among mammalian cell types in its capacity to take up and express naked DNA in the absence of a viral vector or physical carrier, Raz et al. (1994) discovered that mice injected with plasmid DNA encoding the influenza nucleoprotein gene into skin had discrete foci of epidermal and dermal cells, including cells with dendritic morphology, that contained immunoreactive nucleoprotein antigen. A single intradermal administration of 0.3–15 μg of free DNA plasmid induced anti-nucleoprotein-specific antibody and cytotoxic T lymphocytes that persisted for at least 68–70 weeks after vaccination. Intradermal gene administration induced higher antibody titers than did direct gene injection into skeletal muscle and did not cause local inflammation or necrosis. Cytotoxic T cells were also induced. These results indicate that skin cells can take up and express free foreign DNA and promote cellular and humoral immune responses against the encoded protein. Another case for targetting skin rather than muscle has been made by Forg et al. (1998) using a plasmid expressing the β-*gal* gene. In this study the ear pinna was found to be a highly effective site for induction of antibody responses by genetic immunization. Although the muscle revealed the highest expression of gene product, the humoral and CTL responses were lower than in animals injected via the ear pinna. The anatomy of the pinna features two layers of epidermis and dermis separated by cartilage hence doubling the number of professional APCs in comparison with normal skin. A hypothesis that has been postulated to explain why the ear gave better responses over muscle is that the APCs within the ear are directly transfected with DNA then immediately migrate to the draining lymph nodes, therefore allowing for presentation to T and B lymphocytes.

These data reinforce the concept that the types of responses elicited following DNA immunization are dependent on both the identity of the antigen and the route of DNA administration. Furthermore, the use of the β-*gal* gene model has shown that high expression of the encoded gene product does not correlate with immune response intensity.

6.3. Mucosa

The mucosal surface is the first site of contact with inhaled antigens and the development of a strong mucosal as well as systemic immunity is of great importance for protection against infectious agents entering the body via this route. Several studies in animals and humans have provided convincing evidence that protection against a variety of viral and bacterial pathogens can be obtained by intranasal or oral immunization. The major effector substance of mucosal immunity is secretory IgA. While little, if any, IgG antibody reaches the mucosa, some IgA antibody is retained in the blood, and immune cells activated at the mucosal site can mi-

grate (Czerkinsky et al., 1995) and produce IgA antibody both locally and distally. Some tissues, such as the mucosal lining of the respiratory tract and gut, which also serve as barriers against the entry of pathogens, have associated lymphoid tissues that provide high levels of local immune surveillance. Such tissues also contain cells that are specialized for MHC II-restricted presentation of antigens to T helper cells. In fact, if mucosal vaccines can be made to boost IgA output, they may induce protection that is superior to that achieved with conventional i.m. injectable vaccines (Healy, 1990). Mucosal vaccination also raises the intriguing possibility of providing "sterile immunity", where infection is prevented rather than moderated. Such immunity would be very useful with infections such as herpes simplex or HIV where even a small initial infection can cause clinical problems later.

Using a plasmid DNA encoding glycoprotein B(gB) of HSV-1, Kuklin et al. (1997) found that intranasal inoculation produced higher IgA responses than intramuscular injection in mice. Fynan et al. (1993) exploited the high levels of local immune surveillance in mucosal tissues and gave intranasal inoculations (in the form of liquid droplets) of a DNA vaccine to express an influenza virus haemagglutinin glycoprotein. 100 μg of DNA were administered in 100 μl of saline per test site. This route resulted in 76% of the mice being protected against a live virus challenge.

Immunization via the mucosal route offers the advantage that it has the potential to stimulate both mucosal immunity and systemic immunity. It is simple, safe and can be used for the immunization of large population groups. It does not require invasive procedures and avoids the use of needles, an important consideration for safety and economics. A further advantage is the existence of the common mucosal immune system which allows protective immune responses induced at one mucosal site to be expressed at another (Hathaway et al., 1995). While little, if any IgG antibody reaches the mucosae, some IgA antibody does enter the blood and immune cells activated at one mucosal site can migrate to distant sites and so produce IgA antibody both locally and distally.

The ability to deliver DNA in the form of nose drops or even a nasal spray has important implications for the future development and delivery of such vaccines. It must be said that there is no clear indication that one method is truly different or superior to any other because differences in individual pathogens, most notably their method of entry, plays an important role in assessing the route of inoculation.

An ingenious way to deliver DNA orally using transgenic plants has been described recently (Mason et al., 1996; Tacket et al., 1998). Genes encoding Norwalk virus were introduced into both potatoes and tobacco plants. Extracts of tobacco leaf or potato tubers were shown to induce serum IgG and secretary IgA responses specific for Norwalk virus. These results indicate the potential use of transgenic plants for the production and delivery of edible vaccines.

Another consideration in terms of mucosal DNA vaccination, is the induction of tolerance for the treatment of allergic reactions. Roy et al. (1999) have taken the gene from the main peanut allergen *Arah2*, cloned it into an expression vector and administered it orally to AKR/J mice. Dosing with *Arah2* produced an increase in fecal IgA, serum IgG2a titers and a decrease in IgE titers. More importantly the severity of anaphylaxis decreased considerably. The results of this study may hold some prospect of treatment for not only this, but a spectrum of allergies using mucosal DNA immunization.

7. Delivery systems and adjuvants

While of the majority of the experiments conducted to date have used PBS or saline as a diluent for the injected DNA, a great deal of effort is now being applied to the development of delivery vehicles and adjuvants. Such reagents may increase the uptake of DNA, reduce the dose necessary for immunization and enhance subsequent immune responses. Systems currently under investigation are described below.

7.1. Cationic liposomes

Oral immunization, offers ease of administration and acceptability to patients and may be enhanced with the use of a delivery system such as liposomes.

DNA can be entrapped into positively charged (cationic) liposomes, and this technique leads to greatly improved humoral and cell-mediated immunity. Liposomes may act to prevent degradation of DNA when entering the gut mucosa. Modification to the structure of the liposome has been shown to increase the efficiency of DNA binding and uptake. Koltover et al. (1998) produced a two-dimensional, columnar (inverted hexagonal phase) cationic liposome, which allowed easier transfection of mammalian cells in vitro with DNA. This ease of transfection was due to the new complex rapidly fusing and releasing DNA, following adherence to the anionic endosomal vesicles within the host cell. Nevertheless, such approaches reduce the simplicity and advantages that are associated with "naked" DNA immunization, in that there is increased cost, reduced practicality, and the possibility of immunological reactions towards lipid components (Gregoriadis et al., 1997).

7.2. Immunostimulatory oligonucleotide sequences

It was first described more than a decade ago that bacterial DNA, but not eukaryotic DNA, causes immune activation (Sudo et al., 1984; Shimada et al., 1986; Yamamoto et al., 1992). Krieg et al. (1995) were the first to describe the fact that immune activation by microbial DNA results from the content of unmethylated CpG dinucleotides which are common in bacterial DNA but under-represented and methylated in vertebrate DNA. These DNA motifs, consisting of an unmethylated CpG dinucleotide flanked by two 5′ purines (optimally GpA) and two 3′ pyrimidines (optimally TpC or TpT), have been shown to stimulate the innate immune system to produce a series of immunomodulatory cytokines, including IL-6, IL-12 and IFN, and to contribute to the immunogenicity of DNA vaccines (Klinman et al., 1997). The presence of these motifs, also termed immunostimulatory sequences (ISS), was found to be necessary for effective intradermal gene immunization (Sato et al., 1996). As summarized in a recent commentary, ISS in DNA appear to function as Th1 promoting adjuvants (Carson and Raz, 1997). Chu et al. (1997) developed the idea when they demonstrated that immunization of mice with hen egg lysozyme (HEL) protein together with synthetic oligodeoxynucleotides containing CpG motifs (CpG ODN) switched the normally Th2-dominated HEL-specific immune response towards a Th1 type immunity. A recent study on the combined use of alum and CpG ODN to enhance specific immunity induced by a recombinant hepatitis B surface antigen resulted in a very potent humoral response due to the synergistic action of these two adjuvants. Simultaneously there was induction of a CTL-mediated response by the CpG ODN component (Davis et al., 1998).

The use of CpG is rapidly expanding and researchers are currently optimizing usage and investigating its effects on cell types. Sun et al. (1998) have shown that a combination of insect DNA in mineral oil and unmethylated CpG motifs has a stronger adjuvant effect than peptides or proteins injected with complete Freund's adjuvant. Sparwasser et al. (1998) have shown that CpG oligonucleotides trigger maturation and activation of dendritic cells, since the events involved in antigen presentation are essential to the efficacy of DNA vaccines producing immune responses. One can speculate, in the case of DNA vaccination, that CpG motifs may enhance the DC's unique capacity to present to naive T cells and therefore increase presentation of the encoded protein.

7.3. Cytokines

Encoding various cytokine genes in the plasmids of DNA vaccines to increase the immunogenicity and drive specific types of immune response is a strategy that has been used in several studies. Chow et al. (1998) have shown that co-delivery of IL-12 and IFN-γ encoding genes with a DNA vaccine encoding hepatitis B increased the Th1 response (as determined by an increase in IgG2a antibodies) and inhibited the Th2 response. However, when they co-delivered IL-4, a decrease in the Th1 response occurred with a concomitant increase in the Th2 response (assessed by IgG1 antibody production). Increased CTL activity was also noted when IL-12 and IFN-γ were injected with the viral gene. Another cytokine of importance is IL-6 which is critical for end stage differentiation of B cells into IgA-secreting plasma cells. IL-6 also stimulates proliferation of T cells and therefore viruses that enter via the mucosa are dependent upon both local IgA responses

for prevention. Larsen et al. (1998) were able to induce complete protection in mice infected with influenza A when there was co-delivery of IL-6 with a gene encoding haemagglutinin (HA) prior to the challenge, whereas HA alone induced viral clearance but not protection.

Lee et al. (1998) used a bicistronic plasmid to obtain optional responses to hepatitis C (HCV). Their construct contained the E1 and E2 HCV envelope genes as well as the granulocyte macrophage colony stimulating factor (GM-CSF) gene. When tested in Buffalo rats, responses to the viral antigens were greatly enhanced by the presence of the cytokine gene.

7.4. Other systems

Sasaki et al. (1997) used monophosphoryl lipid (MPL) A, an adjuvant of bacterial origin, to enhance DNA vaccination against HIV-1. MPL is derived from *Salmonella minnesota* lipopolysaccharide. It has been shown to activate macrophages and induce IFN-γ and IL-2 to drive a Th1 immune response. Thus, Sasaki et al. (1998a) observed an increase in humoral and cell-mediated responses using MPL combined with a DNA vaccine for HIV-1 relative to DNA alone. The same group also investigated the use of Ubeninex, an anti-cancer modulator (Sasaki et al., 1998b) and QS-21 a highly purified triterpene glycoside saponin isolated from the bark of the *Quillaja saponaria* Molina tree (Sasaki et al., 1998c). QS-21 induces the same cytokines as MPL. In addition this adjuvant was shown to increase CTL activity using ovalbumin and HIV-1 gp120 as model antigens. Furthermore, Ubeninex was shown to induce a Th1 response which suggests the possible enhancement of CTL activity. Therefore it can be seen that adjuvants play an important role in enhancing and manipulating the immune response to increase the chances of pathogen clearance.

Other groups have combined DNA vaccines with other vaccine approaches. For example Schneider et al. (1998) used a particular sequence of pre-erythrocytic antigens of *Plasmodium berghei* expressed in a DNA form in a plasmid vector and a recombinant form in a modified vaccinia vector (Ankara) to induce unprecedented complete protection against a sporozoite challenge by priming with the vaccinia vector and boosting with the DNA construct. Hanke et al. (1998) have also shown a similar effect in an HIV system whereby a plasmid encoding two T cell epitopes (RGPGRAFVTI and SYIPSAEKI) induced IFNγ production and antigen specific CTL responses following priming with a naked DNA construct and boosting with a vaccinia-HIV vector.

Another way to increase the chances of promoting CTL activity has been to enhance the proteasome dependent degradation which induces cellular immunity against targeted antigens (Wu and Kipps, 1997). Rodriguez et al. (1997) showed that co-translational ubiquitination of plasmid encoding viral antigen causes rapid degradation thereby permitting antigen entry and presentation by MHC class I and increased CTL activity, yet no antibody responses were elicited, possibly due to the antigen degrading so rapidly that the B cells could not mount a response.

An alternative method to increase or modulate the immune response is by co-injection of CD154 (the ligand for CD40) which is transiently expressed by T cells upon lymphocyte activation and when co-injected with the model *β-gal* DNA vaccine was shown to augment humoral and cellular immune responses (Mendoza et al., 1997). CD28 co-stimulation has been shown to be essential for antibody and CTL activity. Horspool et al. (1998) showed that by blocking CD28 activity in mice, no humoral or cell mediated responses were seen when the animals were injected with a *β-gal* expression plasmid, yet they retained the ability to up-regulate CTLA4 (a negative regulator of T cell activation). When examining the role of this molecule these authors found that CD28 is regulated by CTLA4 and concluded that there was a need for more research to investigate the functional role of co-stimulatory molecules in DNA vaccination.

8. Practical considerations: advantages vs. disadvantages

8.1. Advantages

Numerous approaches to the delivery of foreign genes into mammalian somatic tissues or organs have been reported, with differing degrees of success. These include using recombinant viral vectors, encapsulation of liposomes and delivery of DNA complexed with specific protein carriers (Fynan et

al., 1995, for review). The problem with viral vectors is that there is always concern over the pathogenic potential of the recombinant vectors. There is also the problem of an immune response being mounted against the actual vector rather than the encoded gene. In addition, such a response, which may be anamnestic, may dominate the response to the recombinant vector. Construction of recombinant viruses, while widely used remains complex. In contrast, cloning a gene into a commercially available plasmid vector with a suitable multicloning site and testing the expression of the gene product by transient transfection can be achieved within a matter of weeks. Cloning the same gene into a vaccinia recombinant virus takes considerably longer. Growing a large batch of plasmid is also a much quicker process than generating an equivalent batch of recombinant vaccinia virus which requires purification by gradient centrifugation and titration by plaque assay. The generation of other viral recombinants is even more time consuming. Needless to say, the expense of producing recombinant viruses is several magnitudes higher than that of generating expression vectors. The speed and economy associated with producing DNA vaccines could be very useful with rapidly mutating viruses, where frequent reformulation of vaccines is required.

Supercoiled circular DNA is stable and easily purified, permitting procedures to be used to remove contaminants which would inactivate a recombinant virus. If plasmid vectors were to be used for mass vaccinations, their high temperature stability would be extremely valuable, especially for developing countries where the cold chain is difficult to maintain (Ertl and Xiang, 1996). Table 2 compares the different types of vaccine strategies currently being investigated.

A major issue to be addressed concerns the relative effectiveness of naked DNA compared to viral vectors in inducing immune responses. Dummer et al. (1995) compared direct intramuscular injection of recombinant plasmids, adenovirus and retrovirus vectors expressing reporter genes, in normal or regenerating muscles of mice. This study showed higher levels of expression produced by the plasmid DNA construct than by the viral vectors. This finding indicates that the use of pure plasmid DNA is a highly efficient means of protein expression and suggests that it is a simple, safe and viable alternative to the recombinant vector constructs.

Aside from being manufactured far more easily than conventional vaccines composed of inactivated pathogens, subcellular fractions or recombinant proteins, the other unique feature of DNA vaccination is

Table 2
Comparison of current vaccine strategies

	Attenuated (Live)	Inactivated	Recombinant protein	Recombinant infectious vector	Synthetic peptides	DNA vaccine
Production	Pathogen is grown under abnormal culture conditions to select a non-virulent type	Virulent pathogen is inactivated by chemicals or gama irradiation	Gene from a pathogen is expressed in bacteria, yeast or mammalian cells requires administration with adjuvant	Genes encoding major antigens especially virulent pathogens are placed into attenuated viruses or bacteria	Peptide sequence that represent immuno-dominant T or B cell epitopes coupled to immunogenic carrier. Expensive to produce	Genes encoding a antigenic protein from a pathogen is inserted into a plasmid vector
Booster required	No	Yes	Yes	Yes	Yes	Possibly
Relative stability	Not very stable	Stable	Stable	Not very stable	Stable	Stable
Type of immune response	Humoral and cell mediated immunity	Mainly humoral responses	Mainly humoral immunity	Humoral and cell mediated immunity	Mainly humoral and cell mediated immunity	Humoral and cell mediated immunity
Reversion	May revert to virulent form	No reversion	No reversion	Vector may revert to virulent form	No reversion	No reversion

the long-lived gene expression sustained. This characteristic permits the presentation of low levels of antigen to the immune system and eliminates the need for booster immunizations (Ulmer et al., 1994).

8.2. Disadvantages

Perhaps the major obstacle facing genetic vaccination of the human population is the possibility of DNA integration into the host genome. This could lead to insertional mutagenesis causing activation of proto-oncogenes or the inactivation of tumor suppressor genes. Autoimmune sequelae including the generation of anti-DNA antibodies might also occur. However the possibility of such events is thought to be low and many studies have investigated the potential for DNA integration. Nichols et al. (1995) looked for any signs of genomic integration in hamstring muscle, lung, kidney, liver, ovary, thymus, spleen, inguinal lymph nodes, mesenteric lymph nodes and blood DNA from mice immunized intramuscularly (quadriceps) with plasmid encoding the nucleoprotein gene of influenza A. Samples were analyzed before and after separation of plasmid DNA by agarose electrophoresis. Free DNA was seen only in the quadriceps and hamstrings and there was no sign of plasmid DNA integration in any of the other tissues examined. Although a watchful eye needs to be maintained for possible adverse reactions, to date it would appear that there are very few practical disadvantages of nucleic acid immunization.

9. Mechanisms

The precise mechanisms involved in the initiation of an immune response following genetic immunization remain to be elucidated. Most studies to date have used intramuscular inoculation of plasmid DNA. Several studies have shown expression of hepatitis B virus surface antigen in myoblasts and myotubules, leading to the suggestion that muscle cells process and present the antigen and stimulate a primary immune response. However, muscle cells express low levels of MHC class I molecules and lack MHC class II expression (Whalen et al., 1995). Interferon is known to upregulate MHC class I expression and hence it may be that during the visible inflammatory response caused by intramuscular injection, IFN collects at the site and locally upregulates MHC expression. Unfortunately, experiments performed to test this theory proved inconclusive (Whalen et al., 1995).

Another theory is that antibody responses are elicited to antigens which are released by myocyte cell death or secretion. These antigens would then be taken up by macrophages and B cells resulting in a T helper dependent antibody response. However a critical element that has often been overlooked when considering DNA vaccination is the role of APCs.

As mentioned previously, sites of injected muscle become infiltrated with inflammatory cells. In the case of MHC class II-restricted CD4 + T cell priming, these APCs would have the opportunity to pick up locally released antigen in the interstitial spaces and carry them to draining lymph nodes where they could be presented to both T and B cells. With regard to MHC class I restricted CD8 + T cells, it may also be worth considering APCs as prime suspects in CTL activation. Theoretically, this does not pose a great problem in terms of intradermal gene vaccination. Within the dermis of the skin, there are many professional APCs such as Langerhans, macrophages and dendritic cells (DC) which are specialized for the uptake and expression of protein antigens. APCs may also take up and express foreign DNA released by dying cells after viral infection. Such a mechanism would facilitate the induction of CTL responses against viruses, especially those that do not directly infect macrophages and dendritic cells.

An experiment performed by Manickan et al. (1997) showed that intramuscular delivery of DC transfected with naked plasmid DNA encoding two proteins of herpes simplex virus (HSV) led to the induction of significantly enhanced levels of resistance to viral challenge. Whereas DC transfected in vitro with DNA induced enhanced immunity, similarly transfected macrophage populations lacked immunogenicity even though plasmid expression occurred in vitro. The enhanced immunity induced by DC-delivered DNA appeared to be associated mainly with an increased Th1 CD4 + T cell response. This finding suggests an involvement for DC as antigen-presenting cells involved in immune responses to intramuscularly administered DNA vaccines. It is

known that within muscle tissue professional APCs are sparse, although they may be recruited to muscle by the local irritation that follows injection. This poses the question: which cell type presents gene encoded antigen to prime the immune system after intramuscular gene vaccination? Although the above experiment shows enhanced immunity with dendritic cells, it is still possible that myocytes are involved in presentation of the expressed antigen. For example, CTL could receive a first signal from peptide/MHC class I complexes expressed by the muscle tissue and a second signal from haemopoietic cells recruited by a local inflammatory response to the site of injection. Therefore, presentation may occur by protein transfer from transfected muscle to professional APC. The primary aim of the experiments reported by Corr et al. (1996) was to determine whether somatic cells at the site of plasmid DNA injection can prime a specific CTL response by presenting antigen in the context of their endogenous MHC complexes or whether presentation is restricted to professional APC of haemopoietic origin. By performing a bone marrow chimera experiment in mice they ensured that only antigen presented by bone marrow derived APCs would be immunogenic, thus excluding immune responses arising from presentation by myocytes. Their results indicated that injection of plasmid DNA encoding the influenza virus NP results in the induction of NP specific CTL restricted to the MHC class I molecules expressed by the bone marrow APC, suggesting that APCs are involved in the presentation of antigen injected intramuscularly. The number of dendritic cells needed to initiate an immune response is minute, and lack of histological evidence for direct transfection of dendritic cells might reflect either that their numbers are below the level of easy detection, or alternatively, that they rapidly leave the site of inoculation and migrate to the draining lymph nodes.

Nonmigratory cells influence the magnitude of the immune response and most importantly, migratory cells alone are responsible for the induction of immunological memory. Primary immune responses are induced by dendritic cells that migrate from the epidermis and antigen-expressing non-migratory cells influence the magnitude of the response. This was demonstrated by gene gun vaccination with a plasmid encoding the CSP protein of malaria (Klinman et al., 1998). Following immunization, the transfected skin was grafted onto a naive recipient inducing an immune response that increased proportionally to skin graft duration. In the case of the mice who had been immunized, graft removal did not reduce B or T cell memory cells. Therefore these data suggest that primary immunity is induced by cells that rapidly migrate from the site of inoculation.

Another factor to consider is the role of the MHC haplotype in these immune responses. Doolan et al. (1996) reported that immunization of BALB/c mice (H-2K^d) with plasmid DNA encoding *Plasmodium yoelii* circumsporozoite protein (PyCSP) is genetically restricted. However, immunization with DNA encoding PyCSP and the hepatocyte erythrocyte antigen (Py HEP17) in combination protected mice of three genetic backgrounds, circumventing the genetic restriction of protection. Tascon et al. (1996) used the gene encoding the 36 kDa antigen from *Mycobacterium tuberculosis* and demonstrated that the genetic background of the host can play an important role in determining the outcome of vaccination. Hsp65 itself carries multiple T cell epitopes that are recognized in the context of a wide variety of murine and human MHC types, and it was effective to different degrees in all the mouse strains. The expression of a small number of such epitope rich antigens may suffice.

10. Clinical trials

As discussed previously, nucleic acid vaccines are being developed for numerous diseases. To date the most advanced trials are for HIV. In animals, including non-human primates, nucleic acid vaccines have induced both HIV-specific neutralizing antibodies and CD8 + CTL (see Johnston and Barry, 1997 for review). Letvin et al. (1997) used an immunization regimen of soluble envelope protein plus a DNA construct encoding HIV_{IIIB} *env* to protect rhesus monkeys against a chimeric HIV-SIV (SHIV) virus challenge. Furthermore, Boyer et al. (1997) immunized animals alternately with constructs encoding gp160 and *gag/pol* from the HIV_{MN} strain. After subsequent boosts the animals were protected against a challenge with HIV_{SF2}. Phase I clinical trials are currently in progress at the University of Pennsylvania and the University of Zurich. These studies are in

collaboration with the Apollon and are using a construct encoding gp120, 41, and *rev* as a therapeutic agent in HIV-infected individuals. A third trial is being conducted by the National Institutes of Health in seronegative volunteers. Other trials currently under investigation include vaccines for malaria and influenza. However, to date, no data have been published.

Calarota et al. (1998) have assessed the efficacy of DNA vaccination in the induction of immune responses in HIV-1 infected humans. Patients were selected for having no or low antibody responses to the antigens that were injected i.e., constructs encoding *nef*, *rev* and *tat* genes of HIV-1. HIV-1 specific CTLs and antigen-specific proliferative responses were measured before, during and after three immunizations over 6 months. The findings revealed that all patients had detectable memory cells and eight developed CTL responses. Some showed CTL activity against gp160. These findings should encourage further studies.

Another clinical trial has commenced using naked DNA as a tumor vaccine. Hawkins et al. (1993) initially injected plasmids encoding immunoglobulin variable (V) genes of anti-idiotypic antibodies into mice. One set of V genes was derived from the idiotype of the murine lymphoma BCL1, a tumor known to be controlled by anti-idiotypic antibodies. Based on this data, a pilot study of idiotypic vaccination for follicular B cell lymphoma sufferers was initiated by generating patient-specific idiotypic DNA vaccines for genetic immunization (Hawkins et al., 1997). The immunogen in the genetic vaccine comprise V genes which will encode a single Fv fragment.

More recently, Wang et al. (1998) have demonstrated that plasmid DNA encoding malaria antigens could be used to induce CTL in healthy humans. This work was carried out as part of a vaccine strategy directed against live-stage *Plasmodium falciparum* and results of these trials are awaited with great interest.

11. Summary

Since its inception little more than six years ago, genetic immunization has advanced very rapidly. To date the technique has proved to be very powerful and has shown utility in a broad range of applications; new uses are being reported almost daily. As we enter the third millennium DNA vaccines may indeed prove to be the third revolution in vaccinology.

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EXHIBIT B

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Suppressive Effects of DNA Vaccines Encoding Heat Shock Protein on *Helicobacter pylori*-induced Gastritis in Mice

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We investigated the effect of DNA vaccines encoding *H. pylori*-heat shock protein A and B (pcDNA3.1-*hspA* and -*hspB*) on inducing immune responses against *H. pylori* in mice. C57BL/six mice aged 5 weeks were immunized by single injection of 10 μg of pcDNA3.1-*hspA* and pcDNA3.1-*hspB* into intracutaneous tissue. Plasmid DNA lacking the inserted *hsp* were injected as a control. Three months after vaccination, significant specific antibodies against *H. pylori* were detected by ELISA in the sera of vaccinated mice. Antibody isotypes were predominantly IgG2a (Th1-like) with pcDNA3.1-*hspA* and mixed IgG1/IgG2a (Th0-like) with pcDNA3.1-*hspB*. DNA vaccination dramatically suppressed colonies of bacteria in stomach of vaccinated mice (28,400 ± 21,600/mm² for pcDNA3.1-*hspA* and 6800 ± 3470/mm² for pcDNA3.1-*hspB*) compared to control mice (128,000 ± 42,200/mm²). Histological analysis of the gastric mucosa demonstrated that the degree of gastritis was significantly lower in the vaccinated mice than in control mice. These results demonstrated that DNA vaccines encoding *H. pylori*-Hsp induce significant immune response against *H. pylori* to decrease gastric mucosal inflammation, indicating that a DNA vaccine can be a new approach against *H. pylori* in humans. © 2000 Academic Press

H. pylori is a spiral-shaped microaerophilic bacterium which colonizes the gastric mucosa of humans. It is the principal cause of chronic active gastritis (1), peptic ulcer (2), and categolized as a class I carcinogen for gastric cancer (3). After infection, it is difficult for a host to eliminate the bacteria from the gastric mucosa in spite of specific immune responses to *H. pylori*. Currently, standard treatment for *H. pylori* infection consists of antibiotics together with a proton pump inhibitor, which results in eradication the bacteria in more than 90% of cases (4).

Because it is feared that antibiotic-resistant strains will emerge (5), there is urgent need to find other approaches for the treatment and prevention of this universal infection. The most attractive new strategy would be the development of an effective vaccine against *H. pylori*. In recent years, administration of plasmid DNA (DNA vaccine) was demonstrated to induce both humoral and cellular immunity, and it has become a promising approach against viral, parasitic, and bacterial pathogens against a variety of animal species. In animal models of human disease, protective responses against HIV (6), herpes simplex virus (7, 8, 9), influenza virus (10, 11), rabies virus (12), malaria (13), leishmaniasis (14), and tuberculosis (15, 16) have been induced by DNA vaccination. To our knowledge, a study of a DNA vaccine against *H. pylori* has not been reported so far. In this study, the protective efficacy of a DNA vaccine against *H. pylori* using HspA and HspB genes was evaluated.

We demonstrate that immunization with plasmid DNA encoding HspA or HspB antigens elicite protective immune responses in a mouse model.

MATERIALS AND METHODS

Bacteria and culture conditions. A mouse adapted *H. pylori*, Sydney strain 1 (SS1) (17) was kindly provided by Professor Adrian Lee (School of Microbiology and Immunology, University of New South Wales, Australia), and was used in this study.

Bacteria were cultured at 37°C in Brucella broth (BBL, Cockeysville, USA) containing 3% FBS (Gibco-BRL, UK) and Skirrow's supplement (SR069E; Oxoid, Basingstoke, UK) under microaerobic conditions with gas generator envelopes (Campy Pak Plus and Gas Pak; BBL, Cockeysville, USA) in gas jars. *E. coli* JM109 which was

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used for all cloning experiments was grown at 37°C in LB medium containing 50 mg of ampicillin per liter.

Preparation of H. pylori-DNA for DNA vaccine. *H. pylori* genomic DNA was prepared as described previously (18). Briefly, bacteria were collected by centrifugation for 2 min at 12,000*g*, resuspended in 400 μl of 10 mM Tris–HCl (pH 8.0) containing 5 mM EDTA, 0.1% sodium dodecyl sulfate and 0.1 mg/ml proteinase K, and incubated for 1 h at 37°C before being treated for 10 min at 65°C with CTAB (10% hexadecyletrimethylammonium bromide in 0.7 M NaCl) and 5 M NaCl. The DNA was phenolized and precipitated with ethanol. After washing twice with 70% ethanol, the DNA was dissolved in 100 μl H_2O, and used directly as a template for the polymerase chain reaction (PCR). The primers for *hspA* and *hspB* were designed according to a previous report (19). The HspA gene was amplified using the forward primer; *hspA*-F (5′ ATTATTGAATTCAATCACAAAAAACACTAGTAC 3′) containing an Asp718 site, and the reverse primer; *hspA*-R (5′ ATTCCTATGGTACCCCGTTTTCTTTAGTTTTAAA 3′) containing an *Eco*RI site. The HspB gene was amplified using the forward primer; *hspB*-F (5′ TTCGTTGAATTCAATGTAGTACGGCGGGTACGG 3′) containing an Asp718 site and the reverse primer; *hspB*-R (5′ TTTAGGGTACCCTGTTGCGGGAGGAAAAGATTA 3′) containing an *Eco*RI site. The PCR mixture (50 μl) consisted of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 4.0 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 1.0 U of AmpliTaqGold DNA polymerase (Perkin-Elmer, Norwalk, Conn.), 20 pmol of each primer, and 1 μl of *H. pylori* genomic DNA. PCRs were comprised of 40 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C. Prior to cycling, the samples were heated at 95°C for 15 min to activate the Taq DNA polymerase. The PCR products were separated using QIAquick Gel Extraction Kit (QIAGEN, CA). The purified *hspA* and *hspB* DNA fragments were each subcloned into TA cloning vector pGEM-T (Promega, Madison, WI) having the sequence of β-galactosidase in the multicloning site following the manufacturer's recommendations (pGEM-*hspA* and pGEM-*hspB*). These plasmids were identified their nucleotide sequences using an Applied Biosystems model automatic sequencer.

Plasmid construction for DNA vaccine. The fragments of Asp718 and *Eco*RI-digested pGEM-*hspA* or pGEM-*hspB* were inserted into the Asp718/*Eco*RI site of pcDNA 3.1 (Invitrogen, San Diego, CA) (pcDNA 3.1-*hspA* and pcDNA 3.1-*hspB*). pcDNA3.1 containing no insert was used for as a control (control DNA). The *hspA* and *hspB* DNA sequences in these fainal plasmids were confirmed again.

Immunization and challenge. Female 5-week-old C57/BL6 mice were used for this study. For immunization, mice were injected intracutaneously with 0.1 ml of saline containing 10 μg of pcDNA 3.1-*hspA*, pcDNA 3.1-*hspB*. Three months after immunization, mice were given one orogastric dose of 10^8 *H. pylori* SS1.

Sample collection. Blood was taken from an orbital vein of each mouse 3 months after immunization, and was used to measure anti *H. pylori*-specific antibodies. Six months after the challenge, the animals were killed and assessed for *H. pylori* infection and gastritis. A 5 mm square piece of the antral portion of each stomach was homogenized in phosphate-buffered saline (PBS), and spread on the *H. pylori* selective agar plates (Eiken Chemical Co., Tokyo, Japan). After 5 days of incubation, bacteria were counted as colony forming units (CFU). The remainder of the specimens were used for grading *H. pylori* colonization and mucosal inflammation by histologic examination.

Histological examination. Each stomach section was embedded in OCT (Tissue-Tek; Sakura Finetech, Tokyo, Japan), snap frozen in liquid nitrogen, and stored at −70°C. Cryosections cut at 5 μm were dried overnight at room temperature, post-fixed in acetone for 10 min, and stained with heamatoxylin and eosin. The degree of antral gastritis was scored using a scale of 0 to 3 modified from that of Marshall and Warren (20) i.e.; 0, intact mucosal lining and essentially no infiltration of the lamina propria with monocytes; 1, mild increase in mononuclear infiltration, localized in the upper half of the mucosa; 2, mononuclear infiltration extending from the surface into the lamina propria resulting in atrophy; 3, marked mononuclear infiltration extending from the surface into the lamina propria and disrupting the structure of the glands and leading to marked atrophy, and/or polymorphonuclear leukocyte infiltration in glands and surface erosions. Each stained specimen was examined blindly by a pathologist. To determine the presence of *H. pylori* on gastric mucosa.

The diaminobenzidine (DAB) peroxidase immunohistochemistry technique was used. In brief, sections fixed in acetone were washed in PBS at 4°C and immunolabeled with rabbit antiserum to *H. pylori* (1:100 dillution; DAKO) for 1 h. Antimouse immunoglobulin-horseradish peroxidase conjugate (1:200 dillution; Histofine simple stain PO; NICHIREI, Tokyo, Japan) was then applied for 30 min. Unbound antibody was removed by washing in PBS and then each section was incubated for 4 min in medium containing 50 mM Tris–HCl buffer (pH 7.6), 0.02% (wt/vol) DAB and 0.01% hydrogen peroxide, washed well in distilled water, counterstained with hematoxylin, air dried, and mounted.

Quantitation of the anti-H. pylori antibody response. An enzyme-linked immunosorbent assay (ELISA) was used to detect anti-*H. pylori* specific IgG and IgA antibody in the sera of mice immunized with DNA vaccine. Flatt bottom 96-well plates (PVC Microtiter; Dynex Technologies, Inc., USA) were coated with 5.0×10^4 *H. pylori* in PBS per well. *H. pylori* antigen were fixed on the bottoms of the wells by centrifugation for 10 min at 100*g*. The plates were washed three times in PBS and then blocked with PBS containing 0.02% NaN_3, 0.1% BSA and 1.0 mg of gelatin per ml over night at room temperature. The plates were then washed three times before the addition of each dilluted sample and then incubated at room temprature for 2 h. The plates were were then washed five times with PBS and then reacted for 2 h with a 1:8000 dilution of horseradish peroxidase labeled goat anti-mouse IgG, IgG1, and IgG2a antibodies (Southern Biotechnology, Birmingham, AL) in Tris saline, pH 7.5 containing 0.1% BSA, washed five times in PBS and developed for 15 min with 3, 3′, 5, 5′-tetramethylbenzicline dehydrochloride (BM blue POD substrate precipitating; Boehringer, Mannheim, Germany). The reaction was stopped by addition of 1N-H_2SO_4 to each well, and plates were read at 450 nm in an automatic microplate reader. The endpoint was defined as the highest dilution of serum giving an O.D. at 450 nm greater than 2 S.D. obtained using nonimmune sera.

Statistical analysis. Data are presented as the arithmetic means ± standard errors (S.E.M.). Statistical differences among groups were identified using one-way analysis of varience and multiple comparisons were performed using the least significant difference method. Differences were analyzed by the Student's *t*-test between two groups with statistical significance set at the 5% confidence interval.

RESULTS

Circulating antibodies. Serum anti-*H. pylori* IgG and IgA antibody responses to DNA immunization were shown in Fig. 1. Immunization with pcDNA 3.1-*hspA* or with pcDNA 3.1-*hspB* significantly induced IgG antibody responses in mice compared to immunization with control DNA without inserts. There were no significant differences in the IgG antibody responses between immunization with pcDNA 3.1-*hspA* or pcDNA 3.1-*hspB* (Fig. 1A). Both pcDNA 3.1-*hspA* and pcDNA 3.1-*hspB* also induced anti *H. pylori* IgA antibody responses in mice while vaccination with control DNA showed no significant IgA response. Mice vaccinated with pcDNA 3.1-*hspA* showed more signif-

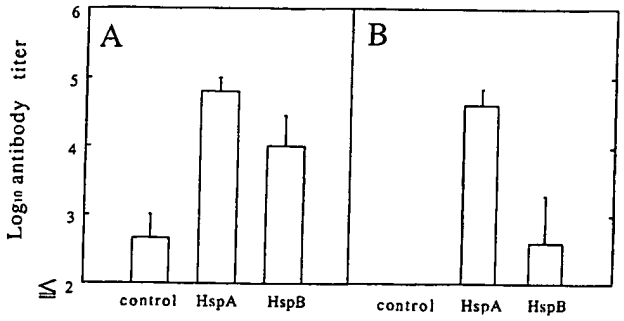

FIG. 1. Serum *H. pylori*-specific IgG (A) and IgA (B) antibody responses (means ± S.E.M.) to pcDNA3.1 (control; $n = 5$), pcDNA3.1-*hspA* (HspA; $n = 5$), and pcDNA3.1-*hspB* (HspB; $n = 5$) administreted intracutaneously.

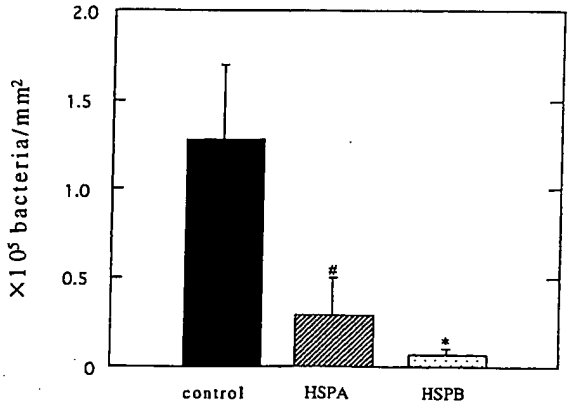

FIG. 2. Protection against *H. pylori* by DNA vaccine. Mice were immunized with pcDNA3.1 (control; $n = 6$), pcDNA3.1-*hspA* (HspA; $n = 4$) to pcDNA3.1-*hspB* (HspB; $n = 5$) by subcutaneous injection. After 3 months, they were challenged with *H. pylori* SS1 (10^8 bacteria). Each stomach was collected 6 months later and dissociated. Their bacterial loads were then evaluated after appropriate dilutions and growth on agar medium. Data are represented as the mean ± S.E.M.; * = $P < 0.01$, # = $P = 0.078$.

icant immune responses in IgA than those vaccinated with pcDNA 3.1-*hspB* (Fig. 1B). To assess the nature of the immune responses to the each plasmid, immunoglobulin isotype profiles were investigated (Table 1). Sera from pcDNA 3.1-*hspA* injected mice contained higher IgG2a antibody titers compaired to IgG1 (Th1-like response), while sera from pcDNA 3.1-*hspB* injected mice had no dominant isotype (Th0-like response).

Quantative analysis of H. pylori colonization. Bacterial CFUs in stomach measured after the DNA vaccines were shown in Fig. 2. Vaccinations conferred significant protection against replication of *H. pylori* in stomachs of B57BL/6 mice following a subsequent challenge but protection with pcDNA3.1-*hspB* (5.3%) was stronger than with pcDNA3.1-*hspA* (22.2%).

Histological findings and analysis of gastritis score. Typical histological findings of gastric mucosa for control and immunized mice with pcDNA 3.1-*hsp* were shown in Fig. 3. In control mice, a lot of inflammatory cell infiltration (Fig. 3A) were observed on the surface and in the gastric mucosa. In vacctinated mice however, much less or very few inflammatory cell infiltration (Fig. 3B) were dettected. Gastric inflammation were semi-quantativly scored and illustrated in Fig. 4. The inflammation scores were significantly lower in both pcDNA 3.1-*hspA* (46.5% reduction) and pcDNA 3.1-*hspB* (16.5% reduction) immunized mice groups than in control group (100%). Colonization with *H. pylori* was verified histologically. There were a lot of bacteria colonies in the mucous layer of stomachs in all control group mice and fewer in HspA and HspB group mice. The values measured by two different methods, CFU and microscopy, almost agreed.

TABLE 1

The Ratio of IgG2a/IgG1 Antibodies

Vaccine	IgG2a/IgG1
HspA	5.3
HspB	1.4

Note. Pooled sera from five mice were analyzed 3 months after immunization with pcDNA3.1-*hspA* (HspA) and pcDNA3.1-*hspB* (HspB) DNA vaccines.

DISCUSSION

It has been shown that immunization with DNA vaccines encoding viral or bacterial antigens can elicit both humoral and cellular immune responses in rodents and nonhuman primates (21, 22, 23). DNA vaccine is easy to produce and purify, and can be stored for a long time because of its biological stability. It is useful for the development of various vaccines and will likely be practical for use in humans in the near future. HspB has been detected on the surface of *H. pylori* in human gastric biopsies (24), and has been previously reported to be a protective antigen in a conventional vaccine (25). HspA may have a role in the interaction between HspB and urease in the chelation of nickel ions, and may be a candidate antigen for a vaccine against *H. pylori* (26).

This study demonstrated humoral responses and the protective ability of DNA vaccines encoding *H. pylori*-HspA or B using the plasmid pcDNA3.1. This plasmid contains the neomycin resistance marker which contains CpG motifs that have been reported to be immunostimulatory (27). The type of immune response reportedly differs with different routes of inoculation such as intramuscular, intravenous, intranasal, and intracutaneous. Intracutaneous injection of plasmid DNA efficiently and reliably primes

humoral immune responses (28, 29). Since humoral immune responses are considered to play an important role in protection against *H. pylori* in stomach (30, 31, 32), we selected the intracutaneous route for immunization and investigated the humoral response and protective efficacy after vaccination with plasmids encoding HspA or HspB. Humoral immune responses were examined in mice vaccinated with either pcDNA3.1-*hspA* or pcDNA3.1-*hspB*. The *hspA* immunized mice acquired a stronger response with both IgG and IgA than the hspB immunized mice (Fig. 1). However, the protective response in the *hspA* immunized group was not significantly stronger compared to that in the *hspB* immunized group (Fig. 2). The IgG antibody isotypes were different in the *hspA* and *hspB* immunized groups, indicating that the *hspA* immunized mice acquired Th1-like immune responses, and that *hspB* immunized mice acquired Th0-like immune responses (Table 1). This

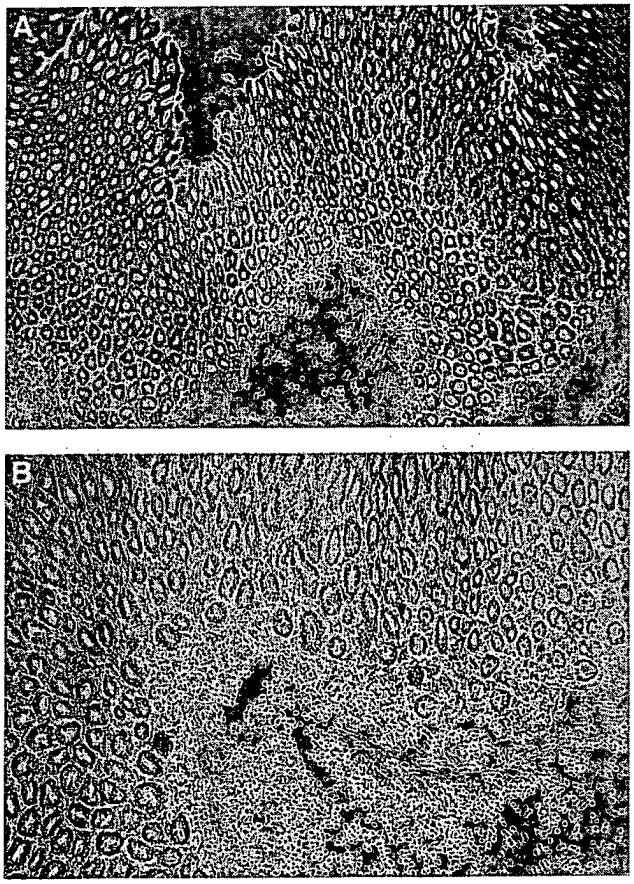

FIG. 3. (A) Animal vaccinated with pcDNA3.1-*hsB*. There are normal epithelial cells both on the surface and within the glands and only minimal mononuclear infiltration (gastritis grade of 0.5). (B) Animal vaccinated with pcDNA3.1 (control). There is intense mononuclear infiltration especially on the surface but also through the lamina propria (gastritis grade of 3.0).

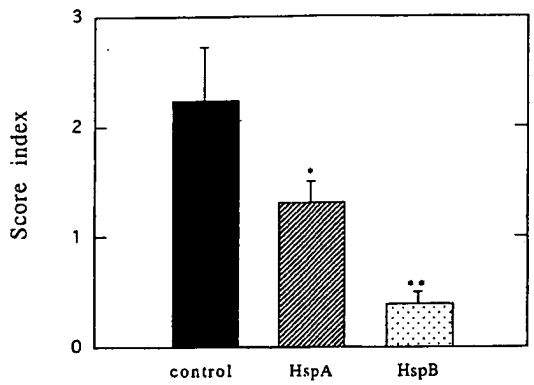

FIG. 4. Effect of DNA vaccination with pcDNA 3.1 (control; $n = 5$) pcDNA3.1-*hspA* (HspA; $n = 4$), and pcDNA3.1-*hspB* (HspB; $n = 5$) on gastritis. Data are represented as the means ± S.E.M. * = $P < 0.05$ and ** = $P < 0.01$.

difference probably led to the result that the protective activity in the *hspA* immunized mice was less than in the *hspB* immunized mice. Considering the immune responses, protective efficacies, and gastritis scores, pcDNA3.1-*hspB* seemed to be a better vaccine than pcDNA3.1-*hspA*. DNA vaccination with pcDNA-*hspB* supressed colonization of *H. pylori* strongly, but did not protect against the infection perfectly. To protect against *H. pylori* infection perfectly, it may be necessary to select more effective DNA antigens and use combination of different plasmid DNA antigens along with adjuvant which have the potential for improving the immune response (33, 34). Because decreasing *H. pylori* in the stomach led to the reduction of gastritis grade in this mouse model, adequate supression without perfect protection against *H. pylori* probably can prevent intense pathogenesis. Therefore, perfect protection against *H. pylori* may not be necessary. We plan to investigate the efficacy of DNA vaccines in the pathogenesis of gastritis, peptic ulcer, and gastric carcinoma using the Mongolian gerbil (35, 36) which is an excellent model for studying *H. pylori* infection, and its relationship to its pathogenesis.

In summary, our results suggest that a DNA vaccine can give significant protection against an orogastric *H. pylori* challenge. The DNA vaccine strategy presented here is easy and should be a promising method for identifying antigens which might be capable of conferring protection against *H. pylori* infection.

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EXHIBIT C

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Immune responses in mice to intranasal and intracutaneous administration of a DNA vaccine encoding *Helicobacter pylori*-catalase

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Abstract

We previously reported that the intracutaneous injection of DNA vaccines encoding *Helicobacter pylori* heat shock proteins elicited specific immune responses, and led to reduced infection in mice. In this study, we constructed DNA vaccine encoding *H. pylori*-catalase (pcDNA3.1-*kat*) and investigated the immune responses to intranasal and intracutaneous administration of pcDNA3.1-*kat*. C57/BL6 mice were immunized intracutaneously with 10 μg of pcDNA3.1-*kat* or intranasally with 50 μg of pcDNA3.1-*kat*. Catalase-specific IgG antibody was detected in the sera of intranasal and intracutaneous immunized mice. Both intranasal and intracutaneous immunized mice were significantly protected from colonization by *H. pylori* and had significantly reduced degrees of gastritis. These results demonstrate that DNA vaccine encoding *H. pylori*-catalase can induce an immune response against *H. pylori*, and that intranasal immunization works as well as intracutaneous immunization.

Keywords: *Helicobacter pylori*; Catalase; DNA vaccine

1. Introduction

Helicobacter pylori is a microaerophillc bacterium which colonizes human gastric mucosa. Infection of gastric mucosa with *H. pylori* to strongly associated with gastritis, peptic ulcers, gastric cancer [1], and mucosal-associated lymphoid tissue lymphoma [2].

Currently, eradication of *H. pylori* by antibiotics together with a proton pump inhibitor, which is successful in more than 90% of cases, is a standard treatment for peptic ulcers [3]. However, there are potential problems concerning antibiotic-resistant strains, so there is urgent need for other approaches to treat and prevent *H. pylori* infection. One new strategy would be to develop an effective mucosal vaccine against *H. pylori*, for which many candidate protein antigens have been reported such as urease [4], heat shock proteins [5], vacA [6], heparan sulphate-binding proteins [7], and lipoprotein 20 [8].

Neutrophils or the other phagocytes in inflamed tissue produce reactive oxygen species to fight pathogenic organisms. Many bacteria produce enzymes such as catalase and superoxide dismutase to protect themselves from oxygen toxicity [9]. Catalase play important roles in a number of environmental situation. In *H. pylori*-catalase may be located throughout the cytosol and in the periplasmic space, and may play a role in protection against oxidative damage caused by processes external to the cell [10]. Orogastric immunization with native and recombinant catalase protein reportedly induce a protective immune response against challenges by *H. felis* and *H. pylori* in a mouse model [11]. More recently, intracutaneous administration of plasmid DNA (DNA vaccine) encoding *H. pylori* heat shock proteins was reported to induce humoral immunity against *H. pylori* infection [12]. There are no other reports of DNA vaccines against *H. pylori*. This study was conducted to evaluate the efficacy of a DNA vaccine encoding

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H. pylori-catalase which was administered intranasally and intracutaneously.

2. Materials and methods

2.1. Bacterial strains and mammalian cell line

H. pylori Sydney strain 1 (SS1) [13] was provided by A. Lee (School of Microbiology and Immunology, University of New South Wales, Australia). *H. pylori* was grown on Brucella broth (BBL, Cockeysville, USA) containing 3% FBS (Gibco-BRL, UK) and Skirrows supplement (SR069E; Oxoid, Basingstoke, UK). After 3 days at 37 °C under microaerobic conditions (CampyPak Plus and GasPak; BBL, Cockeysville, USA), bacteria were harvested and adjusted to a final concentration of 10^9 bacteria/ml. *Escherichia coli* JM109, which was used for all cloning experiments, was grown at 37 °C in LB medium containing 50 mg/l ampicillin. The 293T human embryonic kidney cell line [14] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and penicillin/streptomycin (100 μg/ml of each) and grown in 5% CO_2 at 37 °C.

2.2. Production of DNA vaccine

H. pylori genomic DNA was prepared as described previously [15]. Briefly, bacteria were collected by centrifugation for 2 min at 12,000 × *g*, resuspended in 400 μl of 10 mM Tris–HCl (pH 8.0) containing 5 mM EDTA, 0.1% sodium dodecyl sulfate and 0.1 mg/ml proteinase K, and incubated for 1 h at 37 °C before being treated for 10 min at 65°C with CTAB (10% hexadecyletrimethylammonium bromide in 0.7 M NaCl) and 5 M NaCl. The DNA was phenolized and precipitated with ethanol. After washing twice with 70% ethanol, the DNA was dissolved in 100 μl H_2O, and used directly as a template for the polymerase chain reaction (PCR). The primer for *kat* was designed according to a previous report [16]. The *kat* gene was amplified using the forward primer; 5′-CTGAATTCACATGGTTAATAAAGATGTGAAACAAACCAC-3′ containing an *EcoR*I site, and the reverse primer; 5′-TTCTCGAGTCACTTTTTCTTTTTTGTGTGGTGCATGTCT-3′ containing a *Xho*I site. The PCR mixture (50 μl) consisted of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 4.0 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 1.0 U of AmpliTaqGold DNA polymerase (Perkin-Elmer, Norwalk, CT), 20 pmol of each primer, and 1 μl of *H. pylori* genomic DNA. PCRs were comprised of 40 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1 min at 72 °C. Prior to cycling, the samples were heated at 95 °C for 15 min to activate the Taq DNA polymerase. The PCR products were separated using a QIAquick Gel Extraction Kit (QIAGEN, CA, USA). The purified *kat* DNA fragments were each subcloned into TA cloning vector pGEM-T (Promega, Madison, USA), having the sequence of β-galactosidase in the multicloning site, following the manufacture's recommendations (pGEM-*kat*). These plasmids were identified by their nucleotide sequences using an Applied Biosystems model automatic sequencer. The fragments of *EcoR*I and *Xho*I-digested pGEM-*kat* were inserted into the *EcoRI*/*Xho*I site of pcDNA3.1 (Invitrogen, San Diego, CA, USA) (pcDNA3.1-*kat*). pcDNA3.1 containing no insert was used for as a control (control DNA). The *kat* DNA sequences in pcDNA3.1-*kat* were confirmed again.

2.3. In vitro transfection and expression of catalase

Twenty-four hours before transfection, 2 × 10^5 293T cells per well were seeded into six-well plates, and the mixture of the diluted FuGENE 6 Reagent (Roche, Indianapolis, USA) and pcDNA3.1-*kat* were added to cells. Forty-eight hours after transfection, cells were washed with PBS and fixed with ethanol–acetone (1:1, v/v) for 20 min at room temperature (RT). Expressing catalase were detected by incubating cells for 1 h with a 1:350 dilution of anti-express antibody (Invitrogen, San Diego, CA, USA) whch recognizes the N-terminal peptide sequence of fusion protein expressed from pcDNA3.1-*kat* in PBS containing 1% bovine serum albumin (BSA) at RT. Cells were washed four times in PBS and then incubated for 45 min with a 1:350 dilution of biotinylated goat anti-mouse IgG antibody (Southern Biotechnology, Birmingham, AL) in PBS containing 1% BSA at RT. Cells were then washed four times with PBS and incubated for 45 min with a 1:350 dilution of FITC-conjugated streptavidin (Southern Biotechnology, Birmingham, AL) in PBS containing 1% BSA at RT. After four washes, catalase expression was visualized under a microscope equipped with a 490 nm UV light.

2.4. Immunization and challenge

Female 5-week-old C57/BL6 mice were used for this study. For immunization, mice were injected intracutaneously with 0.1 ml of saline containing 10 μg of pcDNA3.1-*kat* or intranasally with 0.1 ml of saline containing 50 μg of pcDNA3.1-*kat* at days 0, 2 and 4. Three months after the last immunization, mice were challenged once with 10^8 *H. pylori* SS1 cells.

2.5. Sample collection

Collection of blood was carried out on each 2 weeks (0, 2, 4, 6, 8, 10, 12 weeks) from an orbital vessels after the last immunization, and was used to measure anti *H. pylori* antibodies. Six months after the challenge, mice were sacrificed and the stomach of each mouse was collected to assess *H. pylori* infection and gastritis.

2.6. Assessment of H. pylori colonization and histology

Half of the stomach was homogenized in Brucella broth, and spread on *H. pylori* selective agar plates (Eiken Chemical Co., Tokyo, Japan). After 5 days of incubation, colonies were counted to determine the colony forming units (CFU) per gram of stomach tissue. The other half of the stomach was fixed in 10% buffered formalin and 5 μm thick paraffin embedded sections were stained with hematoxylin and eosin to assess the presence of inflammation. The degree of antral gastritis was scored using a scale of 0–3 modified from that of Marshall and Warren [17], i.e. 0, intact mucosal lining and essentially no infiltration of the lamina propria with monocytes; 1, mild increase in mononuclear infiltration localized in the upper half of the mucosa; 2, mononuclear infiltration extending from the surface into the lamina propria resulting in atrophy; 3, marked mononuclear infiltration extending from the surface into the lamina propria and disrupting the structure of the glands and leading to marked atrophy, and/or polymorphonuclear leukocyte infiltration in glands and surface erosions. Each stained specimen was examined by a pathologist unaware of the experiment.

2.7. Purification of recombinant H. pylori-catalase for ELISA antigen

The *kat* genes amplified by PCR using the oligonucleotide primer pairs, the forward primer; 5′-CTGAATTCATGGTTAATAAAGATGTGAAACAAACCACTG-3′ containing an *EcoR*I site, and the reverse primer; 5′-TTCTCGAGTCACTTTTTCTTTTTTGTGTGGTGCATGTCT-3′ containing a *Xho*I site. The PCR fragments were digested by *EcoR*I and *Xho*I and cloned in frame into the GST express vector pGEX-5X-1 (Amersham Pharmacia Biotech Inc., NJ, USA) digested *EcoRI*/*Xho*I (pGEX-5X-*kat*). GST-fusion catalase proteins were produced in *E. coli* JM109 transformed with pGEX-5X-*kat* by induction using isopropyl β-D-thiogalactosidase. Fusion proteins were purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B (Amersham Pharmacia Biotech Inc., NJ, USA). Purified proteins were visualized by SDS-PAGE (Fig. 1). The enzyme activity of the recombinant GST-fusion catalase protein was tested by incubating it with 3% H_2O_2 and checking for the rapid formation of oxygen.

2.8. Quantitation of the anti H. pylori-catalase antibody response

An enzyme-linked immunosorbent assay (ELISA) was used to detect anti *H. pylori* IgG and IgA antibody in the sera of mice immunized with the DNA vaccine. Flat bottom 96-well plates (PVC Microtiter; Dynex Technologies Inc., USA) were coated with 2.5 μg per well of recombinant

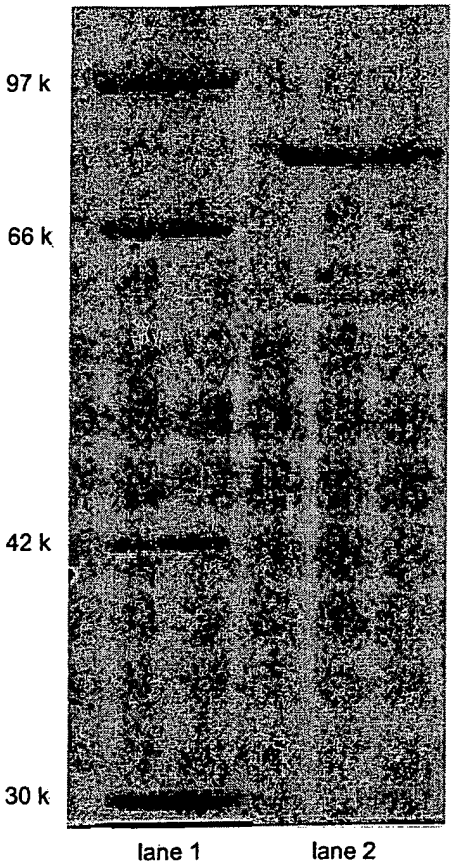


Fig. 1. SDS-PAGE of recombinant proteins used for ELISA antigen. Recombinant proteins were purified by affinity chromatography: lane 1, prestained protein marker and lane 2, GST-catalase (85 kDa) and catalase (59 kDa).

H. pylori-catalase in PBS. The plates were incubated overnight at 4 °C, washed three times in PBS and blocked over night at 4 °C with TBS containing 0.1% NaN_3 and 5% skim milk. The plates were washed three times before the addition of each diluted sample and then incubated for 2 h at RT. The plates then were washed five times with PBS containing 0.05% Tween 20 (PBS-Tween) and reacted for 2 h with a 1:4000 dilution of horseradish peroxidase labeled goat anti-mouse IgG and IgA antibody (Southern Biotechnology, Birmingham, AL) in Tris–saline, pH 7.5 containing 0.1% BSA at RT. After five washes, the color was developed for 15 min with 3, 3′, 5, 5′-tetramethylbenzicline dehydrochloride (TM blue POD substrate precipitating; Boehringer, Mannheim, Germany) at RT. The reaction was stopped by the addition of 1N H_2SO_4 to each well, and the plates were read at 450 nm in an automatic microplate reader. The endpoint was defined as the highest dilution of serum giving an O.D. at 450 nm >2 S.D. obtained with nonimmune sera.

2.9. Catalase neutralization activity

Each concentration of recombinant catalase was incubated with 100-fold diluted serum from vaccinated mice for 1 h at 37 °C before addition of 3% H_2O_2. Catalase activity was assayed quantitatively by measurement of the O.D. at 240 nm which is the absorption peak of H_2O_2 [18].

2.10. Statistical analysis

Comparisons of experimental groups were evaluated by the Student's *t*-test; $P < 0.05$ was considered significant.

3. Results

3.1. Catalase expression in vitro

Immunofluorescence staining of permeabilized 293T cells showed cytoplasmic expression of catalase in cells transfected pcDNA3.1-*kat* (Fig. 2) whereas no fluorescence signal was observed in cells transfected no plasmid.

3.2. Purification and enzyme activity of recombinant catalase

Purified recombinant GST-catalase were visualized by SDS-PAGE (Fig. 1). The detectable two bands shows GST-catalase (85 kDa) and catalase (59 kDa), respectively. It was confirmed that purified recombinant catalase actually have the enzyme activity by checking for the rapid formation of oxygen.

3.3. Serum IgG and IgA responses to H. pylori-catalase

Catalase-specific serum IgG antibodies were first detected 4 weeks after the last immunization, and peaked at the 3 months in both intracutaneous and intranasal immunization groups. At the 3 months after the last immunization, mice immunized by intracutaneous administration of 10 μg pcDNA3.1-*kat* and intranasal administration of 50 μg pcDNA3.1-*kat* had significantly stronger serum IgG antibodies compared to mice immunized with control DNA (Fig. 3A and B, respectively). Mice immunized intracutaneously were induced significantly higher serum IgA antibodies compared to mice immunized with control DNA (Fig. 4A). However, mice immunized intranasally were not induce serum IgA responses in all mice (Fig. 4B).

Anti-serum from mice vaccinated with pcDNA3.1-*kat* showed no catalase neutralization activity because the concentration of H_2O_2 after addition of vaccinated serum and PBS were almost equivalent (Fig. 5).

3.4. Quantitative analysis of H. pylori colonization

Three months after the last immunization, mice were challenged with *H. pylori* and CFUs in stomachs were measured 6 months after the challenge. Both intracutaneous and intranasal immunized mice were significantly protected from colonization by *H. pylori* compared to mice immunized with control DNA (Figs. 6A and 7A, respectively).

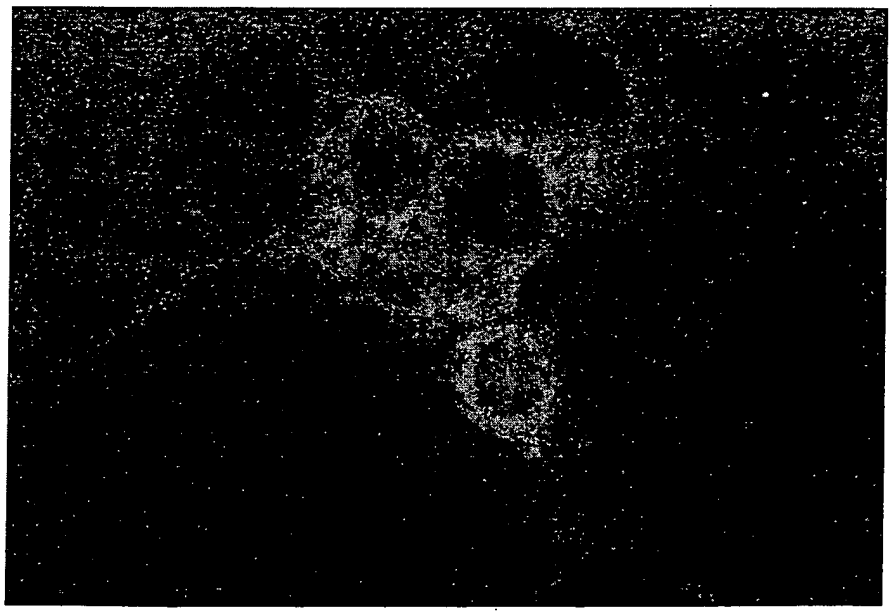
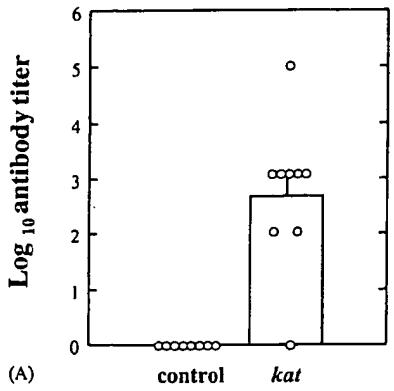


Fig. 2. Expression of catalase protein in cells transfected pcDNA3.1-*kat*. Cells transfected pcDNA3.1-*kat* were fixed and immunostained.



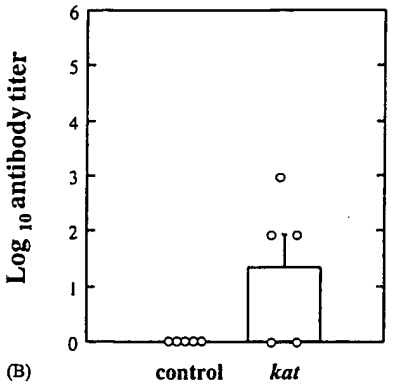
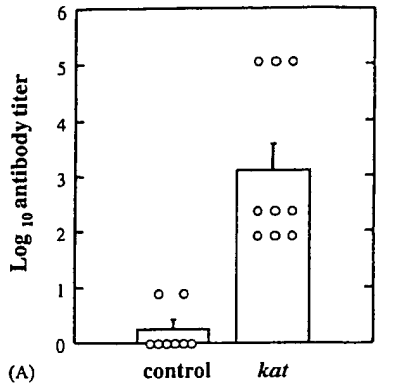


Fig. 3. Serum IgG antibody against to *H. pylori*-catalase. Catalase-specific IgG titers were determined by ELISA. C57/BL6 mice were immunized (A) intracutaneously with 10 μg of pcDNA3.1 (control; $n = 8$) or pcDNA3.1-*kat* (*kat*; $n = 9$) and (B) intranasally with 50 μg of pcDNA3.1 (control; $n = 5$) or pcDNA3.1-*kat* (*kat*; $n = 5$) at days 0, 2 and 4, and blood sample were taken after 3 months. Circles represent serum titers, values are means and error bars indicate S.E.M.



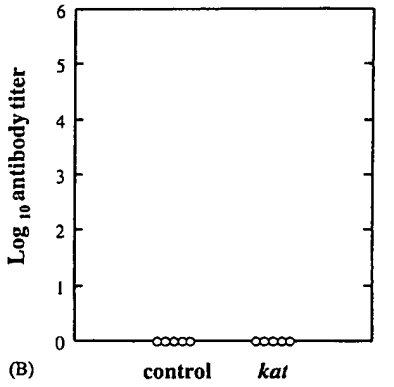


Fig. 4. Serum IgA antibody against to *H. pylori*-catalase. Catalase-specific IgA titers were determined by ELISA. C57/BL6 mice were immunized (A) intracutaneously with 10 μg of pcDNA3.1 (control; $n = 8$) or pcDNA3.1-*kat* (*kat*; $n = 9$) and (B) intranasally with 50 μg of pcDNA3.1 (control; $n = 5$) or pcDNA3.1-*kat* (*kat*; $n = 5$) at days 0, 2 and 4, and blood sample were taken after 3 months. Circles represent serum titers, values are means and error bars indicate S.E.M.

3.5. Histological analysis of gastritis score

Six months after challenge, many inflammatory cells were observed in gastric tissue of mice vaccinated with control DNA. In contrast, there were very few inflammatory cells in mice vaccinated with pcDNA3.1-*kat*. The gastritis score in mice immunized by both intracutaneous and intranasal routes with pcDNA3.1-*kat* was significantly lower compared to mice immunized with control DNA (Figs. 6B and 7B, respectively).

4. Discussion

Mucosal immunization is to exploit common mucosal defense mechanisms [19,20] and induce barrier levels of immunity at multiple mucosal surfaces. This is an important concept for preventing invasion by pathogens which enter the body via mucosa and cause damage at the mucosal site.

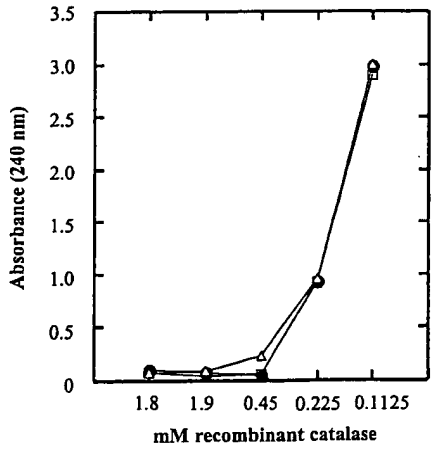


Fig. 5. Catalase activity were determined by measuring spectrophotometrically at 240 nm with 0.3% H_2O_2 after incubating with serum from mice vaccinated with pcDNA3.1-*kat* (△), serum from mice vaccinated with pcDNA3.1 (□) and PBS (●).

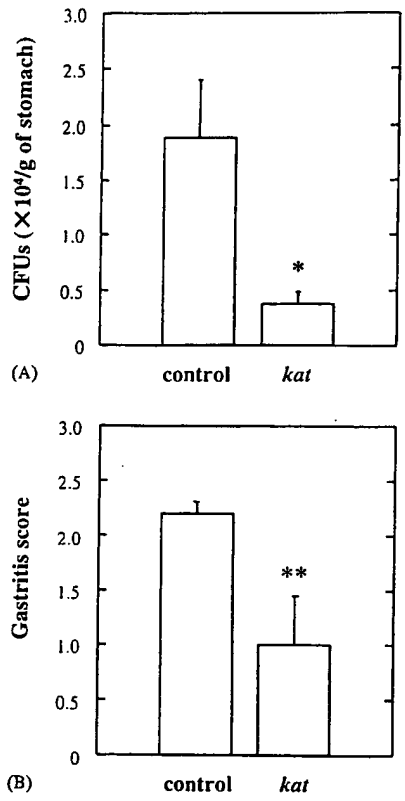


Fig. 6. C57/BL6 mice were immunized intracutaneously with 10 μg of pcDNA3.1 or pcDNA3.1-*kat* at Days 0, 2 and 4. Mice were challenged once with 10^8 live *H. pylori* 3 months after the last immunization. After an additional 6 months, mice were sacrificed and the stomachs were removed and used for assessment of (A) CFUs and (B) gastritis score. Values are means and error bars indicate S.E.M. Asterisk in the figures indicates that the mean value of antibody titer in the *kat* group is significantly higher than that in the control group ($^*P < 0.01$, $^{**}P < 0.05$).

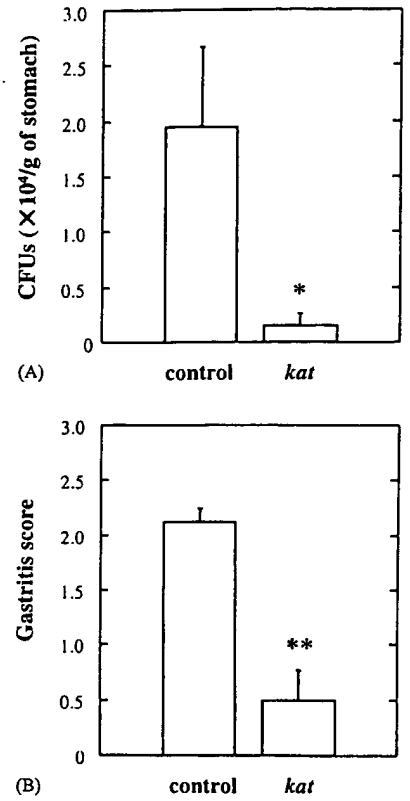


Fig. 7. C57/BL6 mice were immunized intranasally with 50 μg of pcDNA3.1 or pcDNA3.1-*kat* at Days 0, 2 and 4. Mice were challenged once with 10^8 live *H. pylori* 3 months after the last immunization. After an additional 6 months, mice were sacrificed and the stomachs were removed and used for assessment of (A) CFUs and (B) gastritis score. Values are means and error bars indicate S.E.M. Asterisk in the figures indicates that the mean value of antibody titer in the *kat* group is significantly higher than that in the control group ($^*P < 0.05$, $^{**}P < 0.01$).

Intranasal immunization of plasmid DNA induces both systemic and distal mucosal immune responses [21,22]. Nasal administration of plasmid DNA is substantially absorbed into systemic circulation and distributed to different tissues of the body, including the lymph nodes [23]. A previous report on DNA vaccines against *H. pylori* demonstrated that intracutaneous immunization with plasmid DNA encoding HspA or HspB antigens elicits protective immune responses against *H. pylori* colonization of mouse gastric mucosa [12]. This study demonstrated that both intranasal and intracutaneous vaccination with pcDNA3.1 encoding *H. pylori*-catalase induce humoral immune responses, and suppress *H. pylori* colonization and inflammation of gastric mucosa. Intracutaneous immunized mice with pcDNA3.1-*kat* were induced serum IgG and IgA antibodies and suppressed colonization by *H. pylori* compared to mice immunized with control DNA. Above all, mice with high titers of serum antibodies were significantly reduced colonization by *H. pylori* and degrees of gastritis, while mice with low titers were not reduced. Protection against colonization of *H. pylori* seems to correlate with high concentrations of serum antibody. Intranasal immunized mice with pcDNA3.1-*kat* were induced serum IgG antibodies but were not induce serum IgA antibodies. However, all mice immunized with pcDNA3.1-*kat* were significantly reduced colonization by *H. pylori* and degrees of gastritis compared to mice immunized with control DNA. The levels of serum IgA antibody did not correlate with protection of *H. pylori* infection. The difference of serum IgA responses between intranasal and intracutaneous administration may be caused by the different immune responses. The type of immune response differs with different routes of DNA vaccination. In general, intranasal route can activate both Th1-type and Th2-type responses and can induce stronger mucosal IgA and CTL responses in mice [24]. We are considering that the level of mucosal secretory IgA and IgG antibody responses occurred in the stomach is

also important for protection against *H. pylori* infection, and currently investigating the level of IgA and IgG antibody in stomach washes.

Inspite of specific immune responses to *H. pylori* of patients with chronic infection, it is usually difficult for them to eliminate the bacteria from the gastric mucosa. DNA vaccination with pcDNA3.1-*kat* did not give perfect protection against the infection. However, the immune response induced by the DNA vaccine strongly suppressed *H. pylori* gastric colonization, even 6 months after inoculation with the bacteria. These results suggest that if proper immunity against *H. pylori* is induced, *H. pylori* infection can be controlled, which may lead to a reduction of gastritis grade and prevention of serious pathology such as peptic ulcer and gastric cancer. Adequate suppression without perfect protection against *H. pylori* using effective DNA vaccines is probably a new strategy against *H. pylori* associated diseases. It may be necessary to select the best target antigens, routes of vaccination and consider using combinations of different antigens. Adjuvant should also be investigated for improving the immune response to DNA vaccines as reported before [24,25].

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EXHIBIT D

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A plasmid immunization construct encoding urease B of *Helicobacter pylori* induces an antigen-specific antibody response and upregulates the expression of β-defensins and IL-10 in the stomachs of immunized mice

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Abstract

The objectives of this study were to investigate the efficacy of a prototype DNA immunization construct encoding the urease B subunit enzyme of *Helicobacter pylori* (*H. pylori*) for inducing adaptive and innate immune responses in mice immunized via intramuscular or subcutaneous routes and to further explore the adjuvant effects of the CpG motifs in the vector. Antibody, cytokine, and β-defensin profiles were assessed in the stomachs of immunized animals: experiments were terminated 3 months after immunization because there was a significant increase in the anti-*H. pylori* urease B antibody response at Week 6 in mice immunized with the urease B construct. A long lasting expression of IL-10 mRNA was noted. Furthermore, a marked and sustained increase in the mRNA expression of β-defensins was also observed, particularly β1. This study demonstrates that an *H. pylori* urease B DNA construct can induce innate as well as adaptive immune responses in the stomachs of immunized mice. Upregulation of β-defensin gene expression followed immunization and we believe that this is the first report of a DNA vaccine inducing innate anti-microbial responses. Such complex molecular interactions that modulate both innate and adaptive immune responses may be of critical importance in the control of mucosal pathogens, such as *H. pylori*.

Keywords: Vaccine; Gastritis; CpG motifs; Cytokines; Defensins; BALB/c mice

1. Introduction

Helicobacter pylori (*H. pylori*) is well recognized to be a major risk factor for recurrent gastroduodenal inflammatory diseases. Whereas most people develop superficial gastritis, in a small proportion of individuals the infection may progress to greater complications leading to peptic ulcer disease and gastric adenocarcinoma [1]. Two of the main reasons motivating *H. pylori* vaccine studies around the world are the high prevalence of *H. pylori* infection and the disadvantages of antibiotic therapy, including its side effects and its tendency to lead to *H. pylori*-resistant strains.

A new approach in *H. pylori* treatment is the administration of DNA vaccines, which has proven to be highly promising. Recent *H. pylori* DNA vaccines showed significant protection of mice from colonization by *H. pylori* [2–4] and reduction of gastritis [3,4]. DNA vaccines have been considered more technically advantageous than protein vaccines [5]. Although they tend to drive Th1-mediated responses, the outcome of the response can dramatically change depending on several factors, such as the protein expressed by the DNA vaccine and the route of vaccine delivery. An aspect of DNA immunization, which has attracted great interest lately, is the CpG motifs present in DNA vaccines and their ability to trigger humoral and/or cellular responses against bacteria, parasites, and viruses in many preclinical animal models because of their Th1 adjuvant effect [5].

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The most important issue in *H. pylori* vaccine studies and also a major subject of controversy are the type of immune responses required to control *H. pylori* infection. Therapeutic vaccine studies in murine models showed that a switch from an initial T helper type 1 (Th1) cell to a later T helper type two (Th2) response is possibly required for the prevention and eradication of *H. pylori* infection [6–11]. However, recent reports have emphasized that a Th1 response is required for protection [12–15] and a Th2 response is needed to reduce inflammation during *H. pylori* infection [16]. Therefore, the ideal *H. pylori* vaccine should comprise a well conserved and characterized *H. pylori* antigen(s), which will trigger mainly Th2-like immune responses in the stomach of vaccinated hosts without any harmful effects.

Several virulence factors of *Helicobacter,* including the gene products of *ureAB*, *CagA*, *VacA*, and *iceA*, have been characterized in detail and proven to contribute, together with subsequent host defence responses, to the ongoing inflammatory processes that determine the clinical manifestations of *H. pylori* infection. The urease of *H. pylori* is one of the most essential enzymes for virulence and colonization of the gastric mucosa [17], neutralizing the immediate microenvironment of *H. pylori* in the stomach. The urease enzyme consists of two major subunits, UreA (26.5 kD) and UreB (61 kD), at a stoichiometric ratio 1:1 [18]. The antigenic potential of urease has been demonstrated in animal studies with mice [19–21], monkeys [22,23], and humans [24]. *H. pylori* urease subunit B in particular has proven to be more protective and safe than subunit A in mice [21] with no signs of gastritis or other side effects after therapeutic UreB protein vaccination [20].

Recent studies have emphasized the role of epithelial-derived anti-microbial peptides in host innate defence during infection and inflammation. It is still unclear how increased IL-12 and IFN-γ cytokine levels in the gastric mucosa influence host anti-microbial responses against bacteria, such as *H. pylori,* which reside on the luminal epithelial surface. Epithelial cell-derived anti-microbial peptides (β-defensins) may act as effective immune sensors during infections by such bacteria. Studies by Simmons et al. [25] implicate β-defensins as a critical component of effective host defence to *Citrobacter rodentium* infection in the murine gastrointestinal tract (GI). Mouse β-defensin (mβD1), in particular, is closely related to human β-defensin 1 (hβD1) both in sequence and gene organization. It was found to be expressed at low levels in lung, spleen, stomach, and small intestine of mice [26].

In this preliminary report, we analyzed and evaluated the innate and adaptive immune responses induced by a *H. pylori ureB* DNA construct administered intramuscularly and subcutaneously. There are no other reports of immune responses triggered by an *H. pylori* urease DNA construct. The responses were assessed specifically in the stomachs of vaccinated mice and they refer to the upregulated expression of innate (defensins) and adaptive (cytokines) protein molecules.

2. Materials and methods

2.1. Bacteria and culture conditions

H. pylori strain 26695 ($CagA^+$, Vac A^+) was used in this study grown on Blood Agar Base (Oxoid, UK) supplemented with 7% (v/v) lyzed horse blood (TCS Microbiology, UK) and DENT selective supplement (Oxoid, UK) at 37 °C with gentle agitation under microaerophilic conditions in a Variable Atmosphere Incubator (VAIN) (Don Whitley Scientific Ltd, UK). *H. pylori* chromosomal DNA was isolated using a Wizard Genomic Purification Kit (Promega Ltd, UK).

2.2. Cloning and evaluation of the DNA construct

The *ureB* gene (1680 bp) was amplified using *Taq* polymerase (Promega Ltd, UK) in a reaction consisting of 0.2 mM of each dNTP, 0.2 nmoles of each primer and 100 ng DNA template. The primers *ureB F* (5′ ATG AAA AAG ATT AGC AGA AAA GAA 3′) and *ureB R* (5′ CTA GAA AAT GCT AAA GAG TTG CGC 3′) were used for amplification at 94 °C for 1 min, 1 cycle; 94 °C for 15 s, 50 °C for 1 min, 72 °C for 1 min, 25 cycles; 72 °C for 7 min, 1 cycle, and the gene was purified using the Qiaquick PCR Purification Kit (Qiagen, UK). Gene cloning was achieved using pUni/V5-His-TOPO Echo and pcDNA4/His Max-E cloning systems (Invitrogen, UK) in a two-step cloning reaction. First, the gene was cloned into pUni/V5-His-TOPO vector, according to the manufacturer's instructions, and the pUni clones were selected on LB agar plates containing 50 μg/ml kanamycin (Gibco, UK). PCR screening, *Xho I* restriction enzyme digestion and sequencing by use of the ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, UK) confirmed the presence of the gene at the correct orientation and in frame with the vector. The concentration and purity of plasmid DNA were measured by GeneQuantum (Pharmacia, UK) after extraction by using the Qiagen Miniprep Kit (Qiagen, UK). The pUni construct containing the *ureB* gene (donor vector) was recombined with pcDNA4/His Max-E vector (acceptor vector) according to the company instructions. pUni/V5-pcDNA4/His Max-E fusion clones containing the *H. pylori ureB* gene were extracted by using the Qiagen Endofree Plasmid Mega Kit (Qiagen, UK) as described in the manufacturer's instructions (Endotoxin units (EU): <0.1 EU/μg plasmid DNA) and screened by *Pst I* restriction enzyme digestion and PCR screening. Endotoxin levels in the plasmid preparations were tested by using the E-TOXATE kit (Sigma, UK).

2.3. Immunization and sample collection

All animal experiments were carried out with approval from the UK Home Office and according to its regulations and guidelines. Eight-week-old BALB/c mice (Tuck and Sons, UK) in groups of six were used in this study and were housed in a pathogen-free environment. Each mouse was

immunized intramuscularly (i.m.) or subcutaneously (s.c.), with 150 μg endotoxin-free plasmid DNA containing the *ureB* gene (UreB) or vector alone (Vector) on Day 0 and 100 μg at Week 4 after immunization. For i.m. injections, the mice were immunized in the upper thigh and for s.c. immunizations, the mice were injected under the skin of the interscapular area. Blood samples were taken by tail vein puncture at Weeks 0, 2, 4, 6, 8, and 12 after initial immunization and collected in Microvette CB 300 tubes (Sarstedt, Germany). Stomachs were removed on Day 0 and Weeks 6 and 12 after immunization, cut and washed thoroughly in sterile PBS to remove the food contents, snap frozen in liquid nitrogen, and stored at −80 °C until required.

2.4. Quantitation of the anti-Hp antibody response

The antibody levels induced at different times after immunization were assessed by ELISA. *H. pylori* 26695 cells were harvested from DENT agar plates, washed twice with PBS and lyzed by three 30 s sessions of ultrasound, with 30 s cooling periods on ice in between. The supernatant was collected after centrifugation at 13,000 rpm for 20 min and was used to coat the wells of 96-well ELISA plates (Maxisorp, NUNC, UK) overnight at 4 °C (1 μg/well in 0.1 M $NaHCO_3$, pH 9.5) [27–29]. *H. pylori* antigen-coated wells were used for assessing specific and non-specific antibody responses. The plates were washed with PBS containing 0.05% Tween (PBS-Tween) and blocked with 3% bovine serum albumin (BSA) for 1 h at 37 °C. Antigen-coated wells were washed as before and were incubated with serum samples diluted 1:50 in PBS–Tween for 1 h at RT. Bound antibody was visualized by using a polyvalent goat anti-mouse immunoglobulin (IgM, IgG, IgA) horseradish peroxidase conjugate (Sigma, UK), 1:5,000 in PBS–Tween left in antigen-coated wells for an hour. *o*-phenylenediamine was used as a substrate and the optical density readings were read at 450 nm, after the color reaction had developed for 10 min.

2.5. Total RNA extraction

Total RNA was isolated from the mouse stomachs by homogenization of the tissue in TRIzol (monophasic solution of phenol and guanidine isothiocyanate; Invitrogen, UK), followed by $CHCL_3$ extraction and isopropanol precipitation. The concentration and purity of the RNA was determined spectroscopically.

2.6. Semi-quantitative RT-PCR for the expression of cytokines and defensins

Total RNA was reverse transcribed to cDNA by using MMLV reverse transcriptase and buffer (Promega, UK), after being primed with 0.5 μg oligodT (Amersham-Pharmacia, UK) at 70 °C for 10 min and cooled. The reverse transcription reactions were incubated at 42 °C for 1 h followed by heat inactivation at 70 °C for 10 min. The cDNA from each sample was used as a template for subsequent PCR reactions to assess the levels of IFNγ, IL-10, IL-12, mβ1D, and mβ3D. The expression of each molecule was normalized to the expression of GAPDH (glyceraldehyde-3-phosphate-dehydrogenase). The primers (Amersham-Pharmacia, UK) used and the product sizes are indicated in Table 1. Each 50 μl PCR reaction consisted of 25 pmol of each primer, 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 200 μM dNTPs, and 0.5 μ *Taq* enzyme. The defensin genes were amplified at 94 °C for 3 min, 1 cycle; 94 °C for 1 min 30 s, 58 °C for 1 min 30 s, 72 °C for 1 min 30 s, 40 cycles; 72 °C for 5 min, one cycle. The cytokine and GAP genes were amplified at 94 °C for 3 min, one cycle; 95 °C for 45 s, 60 °C for 2 min, 15 s, 40 cycles. PCR products were visualized after electrophoresis on 2% agarose gels, and band intensities were quantified by densitometry (Kodak, Digital Science Electrophoresis Documentation and Analysis System 120, Life-Technologies, UK).

Table 1
Primers used for the amplification and product sizes of IFNγ, IL-10, IL-12, and mβ1D, mβ3D, GAPDH cDNA

Target mRNA	Forward primer 5′–3′	Reverse primer 5′–3′	Size of product (bp)
IFNγ	TGAACGCTACACACT GCATCTTGG	CGACTCCTTTTCCG CTTCCTGAG	460
IL-10	GTGAAGACTTTCTTT CAAACAAAG	CTGCTCCACTGCCTT GCTCTTATT	274
IL-12	AGTGAACCTCACCTG RGACACGCC	GTTTCTTTGCACCA GCCATGAGC	303
mβ1D	GGCTGCCACCACTATGAAA ACTCATTAC	GAGACAGAATCCTCCATG TTGAAGGCA	147
mβ3D	GTCTCCACCTGCAGC TTTTAGCAA	GCATTTGAGGAAAGGAA CTCCACAAC	132
GAPDH	CTACTGGCGCTGG CAAGGCTGT	GCCATGAGGTCCACC ACCCTGCTG	449

2.7. Data analysis

Antibody results represent the average of six or more individually tested mice per group. Statistical significance was established using the Mann–Whitney test and a value of $P \leq 0.05$ was considered significant. RT-PCR results represent the average of three or more individually tested mice per group and are expressed as mean ± S.E.M. Individual experiments were repeated three times.

3. Results

3.1. Antibody response against the H. pylori ureB construct

Each ELISA experiment was performed in triplicate and the total anti-*H. pylori*-UreB antibody response (Ig) elicited via both i.m. and s.c. routes is presented in Fig. 1. The anti-*H. pylori*-UreB antibody response (total Ig) showed a significant ($P < 0.05$) increase 6 weeks after i.m. immunization compared with the response induced by the vector alone at the same time. This specific antibody response against the UreB subunit of *H. pylori* was detected 2 weeks after the boost dose at Week 4 after initial immunization. Immunizations via the s.c. route induced lower levels of *H. pylori*-UreB antibody response compared to the i.m. one. After the 2nd week, the antibody levels induced by the *ureB* construct via the s.c. route were higher than those elicited by the vector alone. However, the antibody responses were almost similar by Week 12 (Fig. 1). Increased antibody levels were noted at Week 6 after immunizations by either route, however, the responses dropped considerably by Week 8. In addition, mice immunized with the vector alone produced a peak response at Week 2, showing a CpG co-stimulatory effect, when administered at the same dose.

3.2. Expression of IFN-γ, IL-10, and IL-12 in the stomachs of i.m. immunized animals

In the present study, cytokine responses were measured at Weeks 6 and 12 and individual experiments were performed three times. IFN-γ expression was detected at Weeks 6 and 12 after immunization in the stomachs of all groups immunized intramuscularly, including the stomachs of non-immunized BALB/c mice (Fig. 2). These results confirm previous studies, which presented low levels of IFN-γ expression in non-infected BALB/c mice used as a control [30]. The amount of IL-12, however, was significantly increased in the group vaccinated with the vector alone at Week 6 after immunization, together with an elevated IL-10 response, showing how the vector has a rapid effect in the induction of cytokines because of the unmethylated

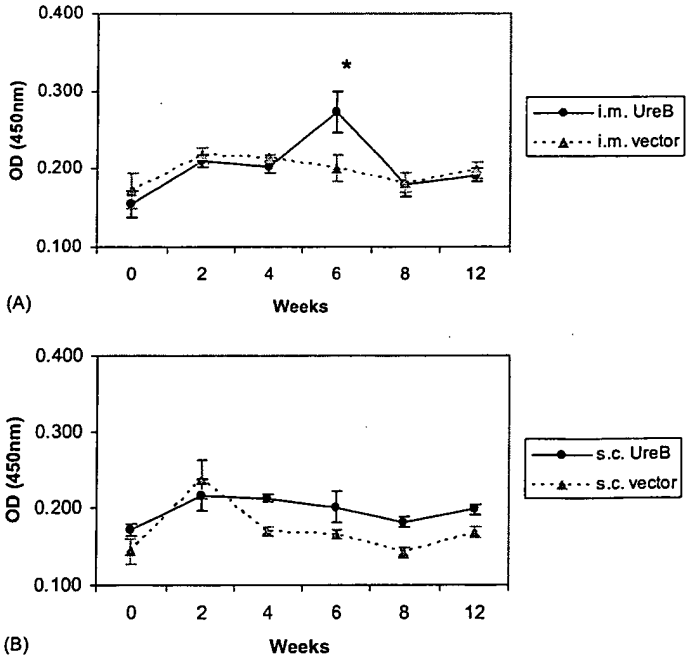

Fig. 1. Anti Hp-UreB antibody response (Ig) induced via the i.m. and s.c. routes. Groups of six mice were immunized with 150 μg DNA at week 0 and 100 μg at week 4. Each point represents the mean OD from the mice in a group ± S.E.M. (A) Total Ig response induced via the i.m. route in the ureB and vector immunized groups. (*) The anti-*H. pylori*-ureB antibody response (total Ig) showed a significant ($P < 0.05$) increase 6 weeks after i.m. compared with the response induced by the vector alone at the same time. (B) Total Ig response induced via the s.c. route in the ureB and vector immunized groups.

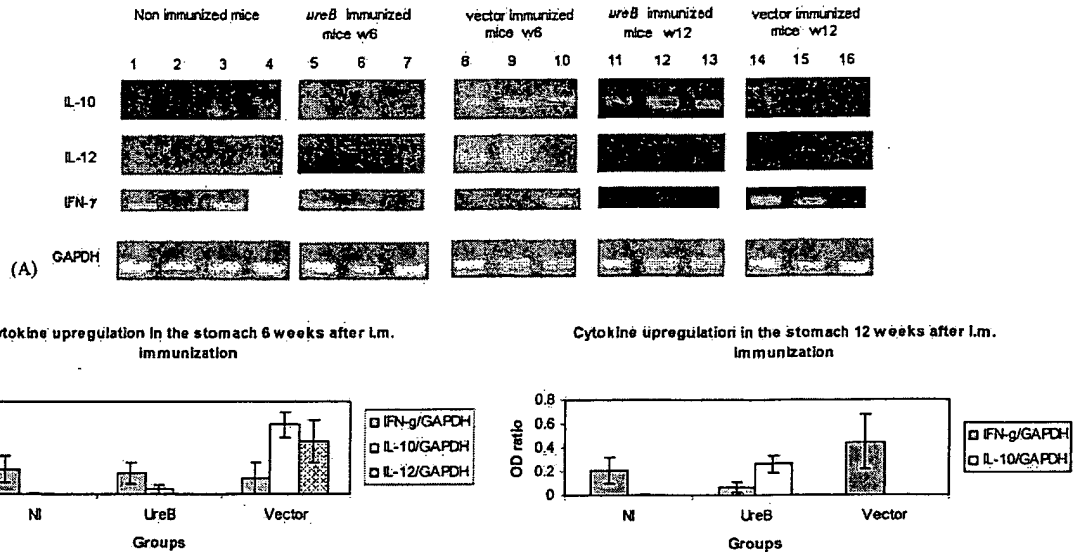

Fig. 2. Expression of IL-10, IL-12 and IFN-γ cytokines in the stomach of i.m. immunized mice. (A) Gastric mRNA from maximum 16 mice was analysed by semi-quantitative RT-PCR and results are shown after visualization on 2% gel electrophoresis gels. (B) Expression was assessed in the stomachs of non-immunized mice (NI), mice immunized with the *H. pylori* ureB DNA construct (ureB) and mice immunized with the vector alone (Vector) at Weeks 6 and 12 after intramuscular immunization. Data are presented as the mean OD ratio of mice in the same group ± S.E.M.

CpG motifs in its structure [31,32]. However, the IL-10 and IL-12 responses triggered by the vector alone were not long lasting, as there was no detectable expression of IL-10 or IL-12 by Week 12 (Fig. 2). Interestingly, increased IL-10 expression was observed in the group immunized with the *ureB* construct up to Week 12.

3.3. Expression of IFN-γ, IL-10, and IL-12 in the stomachs of s.c. immunized animals

IFN-γ expression was detected at Weeks 6 and 12 after s.c. immunization in the stomachs of all groups including the vector alone at both time points (Fig. 3). IL-10 was not

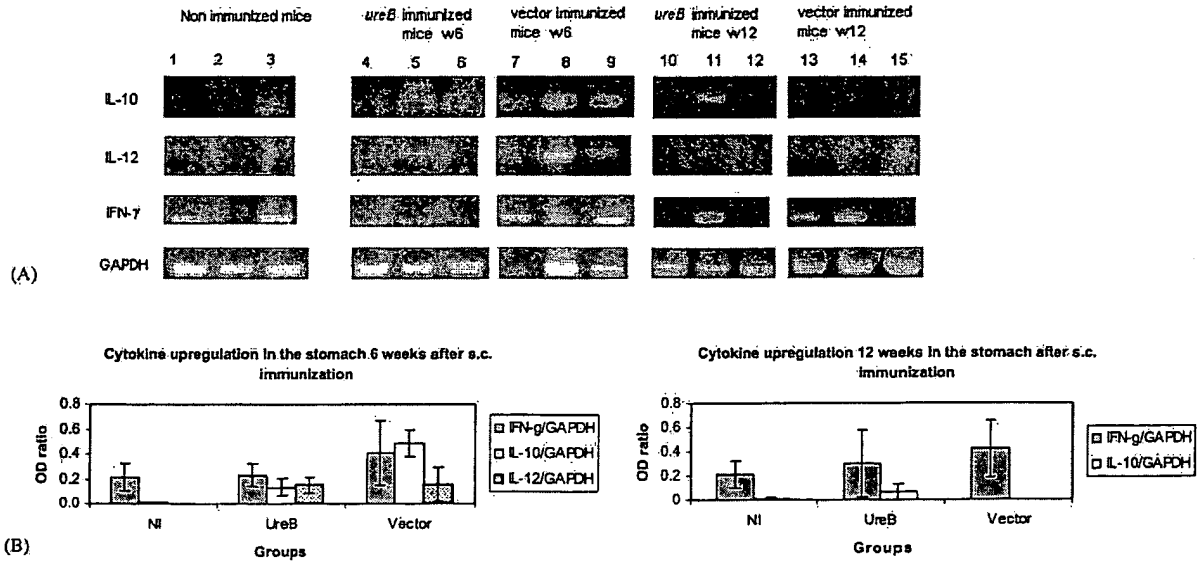

Fig. 3. Expression of cytokines in the mouse stomach after s.c. immunizations. (A) mRNA from maximum 15 mice stomachs was analysed by semi-quantitative RT-PCR and results are shown after visualization on 2% gel electrophoresis gels. (B) Expression was assessed in the stomachs of non-immunized mice (NI), mice immunized with the *H. pylori* ureB DNA construct (ureB) and mice immunized with the vector alone (Vector) at Weeks 6 and 12 after subcutaneous immunization. Data are presented as the mean OD ratio of mice in the same group ± S.E.M.

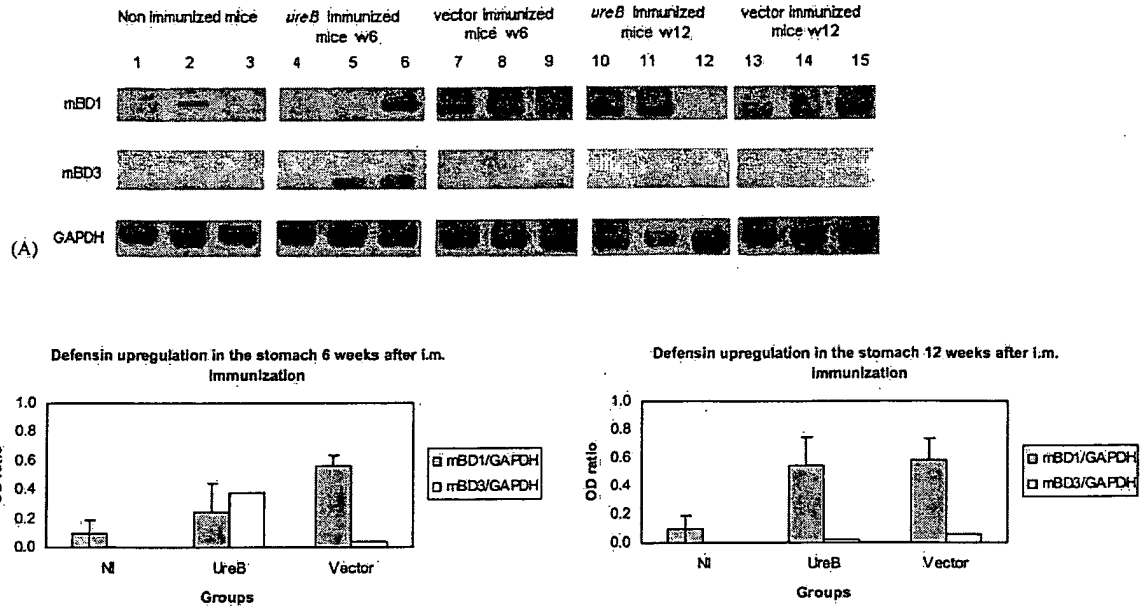

Fig. 4. Upregulation of mBD1 and mBD3 in the mouse stomach after i.m. immunizations. (A) The presence of defensin mRNA was assessed by semi-quantitative RT-PCR in each intramuscularly immunized mouse and results are shown after visualization on 2% gel electrophoresis gels. (B) Defensin expression was assessed in the stomachs of non-immunized mice (NI), mice immunized with the *H. pylori ureB* DNA construct (ureB) and immunized with the vector alone (Vector) at Weeks 6 and 12 after immunization. Data are presented as the mean OD ratio of mice of the same group ± S.E.M.

detectable in the non-immunized groups at Weeks 6 and 12 but was expressed in both immunized groups at Week 6. Similarly to the cytokine response triggered via the i.m. route at Week 12, the response elicited by the vector alone via the s.c. route was not long lasting and had disappeared by Week 12. However, IL-10 expression was still detectable in the group immunized with the *ureB* construct.

3.4. Expression of β1 and β3 defensins in the stomachs of i.m. immunized animals

Defensin expression was assessed at Weeks 6 and 12 and individual experiments were done in triplicate. mBD3 expression seemed to be upregulated in the stomachs of intramuscularly immunized mice with the *H. pylori ureB* construct at Week 6 after immunization (Fig. 4). The induction of mBD1 expression, however, was elevated in both immunized groups by Week 12, indicating that the CpG motifs of DNA constructs induce the expression of defensin peptides in the stomachs of immunized mice.

3.5. Expression of β1 and β3 defensins in the stomachs of s.c. immunized animals

mBD1 expression was upregulated in the stomachs of the mice immunized with the *ureB* construct via the subcutaneous route, but was greater with the vector alone at Weeks 6 and 12 (Fig. 5). mBD3 expression was detectable in the group vaccinated with the vector alone at Week 6, while at Week 12 it was expressed at low levels in both immunized groups.

4. Discussion

In this study, we have demonstrated that a DNA construct encoding the UreB peptide of *H. pylori* is capable of inducing antibody responses in intramuscularly and subcutaneously immunized mice. The antibody responses elicited by the construct via the intramuscular route were low but statistically significant when compared with control animals immunized with the empty plasmid. This finding is very typical of DNA vaccines in general [5,33]. In addition, we noted that immunization induced the upregulation of IL-10 in the stomachs of immunized mice as well as β-defensins (see below).

Several factors dictate the magnitude and effectiveness of the immune response after DNA vaccination including the dose and route of administration as well as the properties of the gene encoding the antigen. For example, intradermal, prophylactic DNA vaccines encoding the heat shock proteins HspA and HspB of *H. pylori* were shown to suppress colonization of the stomach and gastritis in C57Bl/6 mice with the second construct being more effective than the first [3]. Although the pcDNA 3.1-HspA construct induced higher IgG and IgA responses after bacterial challenge, it

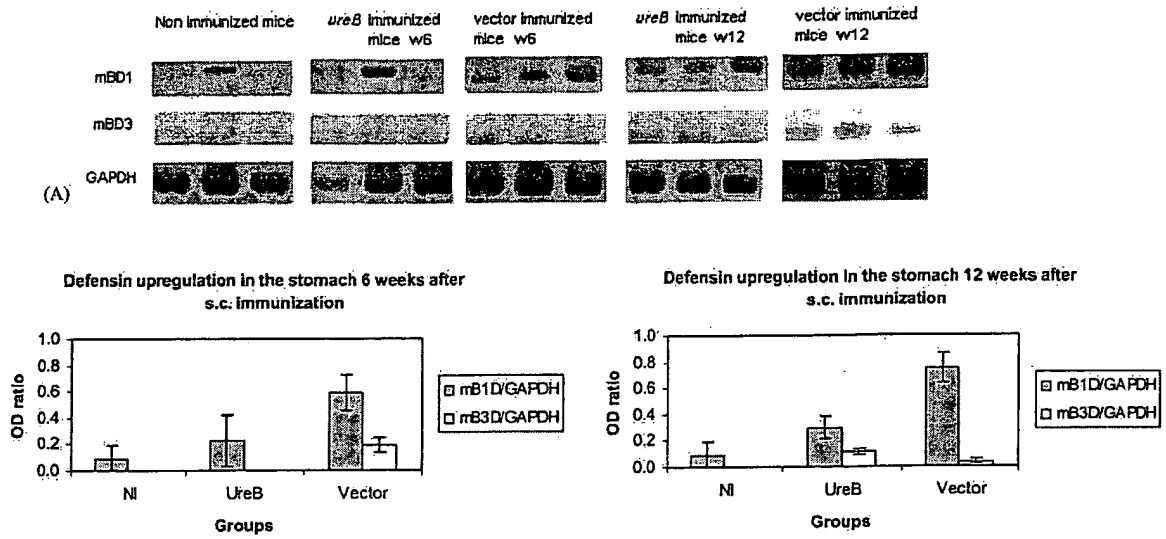

Fig. 5. Expression of β-defensins in the mouse stomach after s.c. immunizations. (A) The presence of defensin mRNA was assessed by semi-quantitative RT-PCR in each subcutaneously immunized mouse and results are shown after visualization on 2% gel electrophoresis gels. (B) Expression was assessed in the stomachs of non-immunized mice (NI), mice immunized with the *H. pylori ureB* DNA construct (ureB) and immunized with the vector alone (Vector) at Weeks 6 and 12 after immunization. Data are presented as the mean OD ratio of mice of the same group ± S.E.M.

conferred a lower level of protection against the bacterium than the pcDNA 3.1-HspB construct, which resulted in a Th0-like response and managed to greatly reduce inflammation in infected mice. This group also achieved a substantial degree of control over gastric re-colonization when an *H. pylori* challenge was given after immunization (intranasal or intracutaneous) with a plasmid encoding the catalase gene [4]. In another study, Ozpolat et al. [2] showed an approximately 100-fold bacterial reduction in the stomachs of mice vaccinated with naked plasmid DNA encoding UreA and B subunits of the *H. pylori* SS1 strain administered intramuscularly.

In the investigations with heat shock proteins and also catalase DNA vaccines [3,4], emphasis has been given to the presence of antibody responses. In our study, a peak of specific anti-*H. pylori*-UreB antibody was noted at Week 6 after i.m. immunization although this trend dropped away sharply, a finding we have noted in a similar study with varicella zoster virus vaccine [34]. Whether this lack of a sustained humoral response would be important in controlling *H. pylori* infection is uncertain, although it is likely that additional booster doses or possibly administration of the DNA formulated with an adjuvant would have increased the specific response [35].

The ability of the *ureB* construct to induce cytokine and defensin production in the stomachs of immunized animals comprises preliminary observations, and more detailed experiments are planned to examine the outcome when different routes of administration are used. The expression of all examined cytokines was upregulated to some extent by the vector alone at Week 6, a phenomenon we attribute to the presence of CpG motifs. IL-10 and IL-12 cytokines were not detected in the stomachs of vector immunized mice 12 weeks after both routes of immunization, indicating that the vector had a rapid and not a sustained effect in the production of these cytokines. Indeed the immediate effect of the CpG motifs in the vector during DNA immunization has been previously demonstrated [31,32].

The *H. pylori ureB* construct seemed to suppress Th1-type cytokine responses favoured by the vector only, since no expression of IL-12 was noted in the stomachs of the *ureB*-immunized group at Week 6 post-i.m. immunization, and the expression of IFN-γ was significantly lower at Week 12 post-i.m. immunization, compared with the response induced by the vector alone. Also, the expression of IL-10, an anti-inflammatory cytokine seemed to persist with time in the *ureB* group immunized via both parenteral routes. IL-10 has proven to be an important regulator of the inflammatory response to *H. pylori*, since IL-10-deficient mice had developed severe hyperplastic gastritis [36].

It is believed that a Th2 response eliminates the infectious organism during parasitic and bacterial infections and moderates any pathologic consequences of the earlier Th1 response. However, researchers must take into consideration that Th1- or Th2-only responses occur in cases of dysfunctional immunopathology and not in the normal function of the immune system against pathogenic organisms. Therefore, a balanced immune response between the two T helper cells may lead to infection resolution, rather than the induction of a specific Th cell pathway [37].

There is increasing evidence to support a crucial role for endogenous anti-microbial peptides in host defense during

infection and inflammatory episodes. Biragyn et al. [38] noted β-defensin production after mice were immunized via the intradermal route with an HIV gp120 construct fused with various chemoattractants including β-defensin 2. The present study, however, is the first to characterize the gene expression of epithelial β-defensins in the murine stomach after DNA vaccination without using any adjuvant or chemoattractant. We wish to emphasize that we restricted the measurement of defensin upregulation to the PCR technique because the reagent availability for performing tissue studies is still very limited.

Interestingly, the expression of constitutive mβD1 was found to be upregulated by the vector alone 6 weeks post-vaccination via both parenteral routes and remained at higher levels in both groups of treated mice when compared with control 12 weeks post-i.m. vaccination. In contrast, the gene expression of mβD3 reached significant levels only in the group of mice given the *ureB* construct 6 weeks after i.m. vaccination. The expression of mBD1 was also found to be upregulated mainly by the vector alone up to 12 weeks after i.m., s.c. immunizations (Figs. 4 and 5) probably due to the adjuvant effect of the unmethylated CpG motifs and the upregulation of cytokines. Several factors may contribute to the observed pattern of defensin gene expression. The CpG motifs present in the vector are known to have potent adjuvant properties [39,40], and such sequences may modulate defensin gene expression via activation of the Toll 9 receptor. Therefore, we present the hypothesis that DNA vaccines are likely to modulate β-defensin expression in the stomachs of immunized mice, most likely due to the adjuvant effect of CpG motifs and the changes in the cytokine micro-environment.

The identity of the molecular mechanism(s) involved in defensin gene expression remains unclear; however, the increased expression of Th1 and Th2 cytokines in the various groups may directly or indirectly regulate defensin gene expression. Recently, Simmons et al. [25] showed augmentation of mβD3 expression in IFNγ and IL-12 C57Bl/6 mice infected with an enteric non-invasive pathogen, *C. rodentium*, suggesting a role for Th1 cytokines in mβD3 regulation. Although an increase in Th1 cytokines was observed in the present study, a direct correlation between cytokine level and defensin gene expression could not be established, mainly due to the small number of samples. The use of different strains of mice in the two studies makes direct comparisons difficult. Furthermore, different sites (stomach versus colon) in the GI tract may exhibit inherent differences in their ability to mount an anti-microbial response.

Overall, this study demonstrated that the vector had a different immune profile from the *ureB* construct and that the presence of the *ureB* gene in the vector altered the immune profile of the plasmid via both routes of administration, possibly because the UreB protein expressed by the vector influences the cytokine-inducing effects of the CpG motifs in the plasmid (Figs. 2 and 3). This alteration in the vector's immogenicity proved to be important for bacterial reduction in *ureB* immunized mice via the intranasal route (Hatzifoti et al., unpublished data). In addition to this observation it was found that both parenteral (i.m. and s.c.) routes managed to induce local cytokine and defensin immune responses in the stomachs of mice immunized with the vector and the *ureB* plasmids. It can, therefore, be concluded that the plasmid construct encoding the UreB antigen could be further explored in future *H. pylori* vaccine studies, possibly in combination with other immunogenic antigens.

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